

**INNATE IMMUNE RESPONSES ACTIVATED BY THE ADJUVANT POLY [DI
(SODIUM CARBOXYLATOETHYLPHENOXY)PHOSPHAZENE](PCEP) IN PIGS**

A Thesis Submitted to the College of
Graduate and Postdoctoral Studies
in Partial Fulfillment of the Requirement of the Degree of
Doctor of Philosophy
in the Vaccinology and Immunotherapeutic Program
in the School of Public Health
University of Saskatchewan
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ABSTRACT

Adjuvants are critical components of vaccines because they enhance antigen-specific immune responses to protect against disease. However, the mechanisms of action (MOA) of most adjuvants are not well understood and they particularly are under-investigated in large animal species including pigs and cattle. This knowledge gap may limit our ability to design effective vaccines for livestock. Understanding the mechanisms by which adjuvants mediate their effects could provide critical information on how innate immunity influences the development of adaptive immunity. Furthermore, knowledge on the MOA of adjuvants may inform vaccine the safety. In the present investigations, we studied the MOA of the experimental adjuvant polydi(sodium carboxylatoethylphenoxy)phosphazene (PCEP) in pigs. First, we administered PCEP by intradermal (i.d.) injection into pigs and assessed its impact on the expression of select immune response genes known as ‘adjuvant response genes’ over time. We observed that PCEP induced the expression of chemokine (C-C motif) ligand 2 (CCL-2), proinflammatory cytokine interleukin (IL)-6, IL-13 and macrophage scavenger receptor 1 (MSR1) genes at the site of injection. Next, we evaluated whether these gene expressions translate to protein transcription by accessing local and systemic production of cytokines after intradermal injection of PCEP into piglets and whether the cytokines produced induces recruitment of immune cells at the site of injection and in the draining lymph nodes. We observed that, at the site of injection, PCEP induced increased production of IL-1 β and IL-13 cytokines, increased cellular infiltration of macrophages, T and B cells, and other leucocytes especially neutrophils as well as showing necrotic debris which might cause release of damage associated molecular patterns (DAMPs) and activate the inflammasome. In the draining lymph nodes, the cytokines IL-1 β and IL-6 were elevated and there was increased leucocyte infiltration.

No changes in cytokine levels were detected in the blood after PCEP injection indicating that the immunostimulatory effect of PCEP is local but not systemic.

Because i.d. injection of PCEP induced signs of necrosis (cell death), we investigated whether reduction of the adjuvant dose reduced tissue damage without negatively impacting antigen-specific immune responses. We conducted two studies to address this issue. In the first study, we injected piglets i.d with varying doses of PCEP alone as follows: 500 µg, 100 µg, or 20 µg PCEP into piglets and evaluated the inflammatory responses. The four parameters evaluated were granuloma formation, lymphocytic infiltration, necrosis, and suppurative inflammation at the injection site and the draining lymph nodes over 14 days. When PCEP was injected alone, we observed that only 500 µg consistently induced significant necrosis and suppurative inflammation. However, the medium dose (100 µg) PCEP did induce significant skin granulomas and lymphocyte infiltration, where as the only significant response induced in the skin by the lower dose (20 µg) PCEP was lymphocyte infiltration. In the draining lymph nodes, only 500 µg PCEP significantly higher suppurative inflammation. No necrosis or granuloma was observed in the lymph nodes in all the doses. Thus, the high dose of adjuvant triggered the most significant pathological signs of tissue damage at both sites (skin and draining lymph nodes). In the second study, we co-injected i.d varying doses of PCEP 500 µg, 100 µg, 20 µg, or 4 µg co-formulated with inactivated swine influenza virus (SIV) H1N1 antigen and measured the four parameters of inflammatory response (granuloma formation, lymphocytic infiltration, necrosis, and suppurative inflammation) at days 20 and 41 after a single injection at each sites and also assayed SIV H1N1-specific antibody titers. We observed that the highest dose of the adjuvant PCEP (500 µg) induced significant inflammatory responses in 3 of the 4 parameters assessed at day 20, and by day 41, this high dose of adjuvant had caused significant tissue response in all the

four parameters assessed. Interestingly, only 500 µg of PCEP induced significant granuloma formation and necrosis (the more severe lesions) at both time points. Of the lower doses, 100 µg and 20 µg of PCEP both induced significant lymphocytic infiltration compared to 4 µg of PCEP, SIV and PBS groups. The lowest dose, 4 µg of PCEP did not induce any significant inflammatory response in any of the four parameters assessed. When SIV H1N1-specific antibody titers were assessed in immunized animals, only 500 µg, 100 µg and 20 µg of PCEP induced significant antibody responses when compared to SIV H1N1, confirming that these doses of PCEP had adjuvant activity. In contrast, 4 µg of PCEP did not induce any significant SIV H1N1-specific antibody titers, indicating that this dose did not have adjuvant activity. These results suggest that induction of inflammatory responses at the site of injection is necessary for adjuvant activity. However, not all responses assessed may be required for adjuvant activity as induction of very severe inflammatory responses was not associated with any additional increase in antigen-specific antibody titres. Thus, the quality of the inflammatory response is important but severe inflammation was not beneficial to antigen-specific immune responses

Together, our data indicate that in pigs, the adjuvant PCEP stimulated early inflammatory responses at the injection site, creating an immunocompetent environment that led to activation of innate immune response genes, production of cytokines and chemokines, and recruitment of various immune cells. These events contribute to the adjuvant activity of PCEP. We propose a MOA model whereby PCEP induces tissue damage at the site of injection and the subsequent release of damage associated molecular patterns (DAMPs) which may activate the inflammasome and contribute to increased immunogenicity of the co-administered antigens.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisors Dr. George Mutwiri and Dr. Heather L. Wilson for their guidance, support, constructive criticism and encouragement. Their enthusiastic and valuable assistance throughout my PhD program has been a rewarding and enjoyable experience that I cannot regret. I also am grateful to the members of my advisory committee Dr. Suresh Tikoo (chair), Dr. Baljit Singh, Dr. Yanyun Huang, and Dr. Susantha Gomis for their keen interest and valuable suggestions throughout the course of this research.

To members of my advisory committee:

“It is not the critic who counts; not the man who points out how the strong man stumbles, or where the doer of deeds could have done them better. The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood; who strives valiantly; who errs, who comes short again and again, because there is no effort without error and shortcoming”— Theodore Roosevelt

I thank all the animal care staff especially Dr. Stew Walker and Ms. Jan Erickson for their kind cooperation and timely help during animal experiments. I am very thankful to Ken Lai and Donna Dent for their valuable technical help and discussions throughout this project. Special thanks goes to Prairie Diagnostic Services laboratory team and Dr. Yanyun Huang for their help in histological preparation and analysis. I sincerely acknowledge all the members of VIDO laboratory A121 especially Ken Lai and everyone at VIDO-InterVac who rendered their help during my research, which made my life at the institute enjoyable.

Some special words of gratitude to my friends who have always been a major source of support when things got a bit discouraging: thanks for always being there for me. I am also grateful to my family members for their endless support and love.

Finally, I would like to thank the Natural Sciences and Engineering Research Council of Canada, Agriculture Development Fund (Saskatchewan), Alberta Livestock and Meat Agency and Vaccinology and Immunotherapeutics Program, School of Public Health for their financial support.

DEDICATION

To everyone who unconditionally supported me throughout my education life

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LIST OF ABBREVIATIONS

AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
APC	Antigen presenting cell
AS03	Adjuvant system 03
AS04	Adjuvant system 04
ATP	Adenosine triphosphate
BAFF	B-cell activating factor
BCR	B cell receptor
BRSV	Bovine respiratory syncytial virus
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CIITA	MHC class II transcription activator
CpG ODN	Cytosine-phosphate-guanosine oligodeoxynucleotides
CLIP	Class-II-associated invariant chain peptide
CLRs	C -type lectin receptors
CMI	Cell-mediated immunity
CR	Complement receptor
Ct	Cycle threshold
CSF3	Colony-stimulating factor 3

CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine C-X-C receptor
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsRNA	Double stranded RNA
DTaP	Diphtheria, Tetanus, acellular Pertusis vaccine
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FDCs	Follicular dendritic cells DCs
GC	Germinal centre
HAU	Hemagglutinating unit
HBs-Ag	Hepatitis B surface antigen
HBV	Hepatitis B virus
HDP	Host Defense Peptide
HEV	High endothelial venules
Hib B	Haemophilus influenzae type B
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus

ICAM	Intercellular adhesion molecules
IFA	Incomplete Freund's Adjuvants
IFN	Interferon
IgG	Immunoglobulin
IL-1 β	Interleukin-1 beta
IL	Interleukin
i.m.	Intramuscular
i.d	Intradermal
iNKT	Invariant natural killer T cells
ISCOMs	Immune stimulating complex
LFA-1	Leukocyte function associated antigen 1
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MDP	Muramyl dipeptide
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility
MMP	Matrix metalloproteinases
MOA	Mechanisms of action
MPL	Monophosphoryl lipid A
MMR	Measles, mumps, and rubella
MSR1	Macrophage scavenger receptor 1
NAIP	Neuronal apoptosis inhibitor protein
NALP3	NACHT, LRR and PYD domains-containing protein 3

NFκB	Nuclear factor κB
NK	Natural killer (cells)
NKT	Natural killer T cells
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PBS	Phosphate buffered saline
PAMPs	Pathogen associated molecular patterns
PCEP	Poly[di(sodium carboxylatoethylphenoxy)phosphazene]
PCPP	Poly[di(sodium carboxylatophenoxy)phosphazene]
pDNA	Plasmid DNA
PZ	Polyphosphazenes
PCR	Polymerase chain reaction
PEG	Poly(ethylene glycol)
PMN	Polymorphonuclear
PP	Peyer's patches
PRR	Pattern recognition receptor
PT	Pertussis toxoid
PYD	Pyrin domain
qRT-PCR	Quantitative real-time PCR
RIG	Retinoic acid-inducible gene 1
RLR	RIG-1 like receptors

rRNA	Ribosomal RNA
SIV	Swine influenza virus
ssRNA	Single stranded RNA
TAP	Transporter associated with antigen processing
TBST	Tris-buffered saline plus 0.05% Tween-20
TCR	T cell receptor
TCM	T central memory cells
TEM	T effector memory cells
T _{FH} cells	T follicular helper cells
tgD	Truncated glycoprotein D
TGF- β	Transforming growth factor beta
Th1	T-helper lymphocyte type-1
Th2	T-helper lymphocyte type-2
TIMPs	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
TNF- α	Tumor necrotic factor alpha
TP1	Telomerase-associated protein
Tregs	T regulatory cells
TriAdj	Triple adjuvant combination
VCAM	Vascular cell adhesion protein

CHAPTER 1: LITERATURE REVIEW

1.1 The immune system

The immune system is a framework of cells, tissues, and organs that cooperate to shield the body against infection by "outside" invaders (Medzhitov & Janeway, 1997). These foreign invaders are principally microorganisms such as bacteria, parasites or fungi. To ensure that individuals are adequately protected against infections, the immune system should be able to perform the following function: 1) Immunological recognition of the pathogen or antigen, 2) Containment of the infection and possibly killing of the infectious agent through effector functions, 3) Immune regulation that limits harm to the host, 4) Establish immunological memory. Immunity is divided into two parts dictated by the speed and specificities of the responses induced (Abbas et al., 2014b). These are named the innate and the adaptive immune responses, in spite of the fact that there is much association between them.

1.1.1. Overview of innate immunity

The innate immune system is present since birth and it is the initial response by the body to eliminate microbes and to prevent infection (Akira et al., 2001; Kawai & Akira, 2010). Innate immunity is an antigen-nonspecific defense mechanisms that is activated immediately or within hours after exposure to microbes and antigens (Medzhitov, 2001).

Innate immune cells such as neutrophils, epithelial cells, fibroblast and monocytes and tissue phagocytic cells such as macrophages recognize pathogen-associated molecular patterns (PAMPs) present in infectious agents which bind to pattern-recognition receptors (PPRs) expressed by mammalian cells (Akira et al., 2001). Pathogenic microorganisms are remarkably different but they have some basic patterns in segments of their structures, for example, the cell

wall or nucleic acids. PRRs can react to PAMPs including proteins, lipids, and sugars, DNA and RNA. Various PAMPs have been depicted for some classes of pathogenic microorganisms, for example, Gram-negative organisms, Gram-positive microbes, viruses, fungi, and protozoa as described in Table 1. The cooperation among PRRs and PAMPs include the initial reactions to outside or foreign substances and are basic for pathogen regulation and amplification of the full repertoire of the immune response (Mogensen, 2009). The innate immune cells release chemokines and cytokines that trigger inflammation to contain the infection and immune cell recruitment to kill the invading pathogen. Recruited defense cells include phagocytic cells such as blood-derived neutrophils, macrophages, mast cells, basophils, eosinophils and natural killer (NK) cells are recruited and release inflammatory mediators that augment more inflammation and recruitment of immune cells (Takeda & Akira, 2005)

1.1.1.1 Innate immune receptors and their respective ligands.

PRRs are developmentally moderated receptors on different types of innate immune cells and are equipped for reacting to PAMPs (Akira et al., 2001). PRRs can reside on the cell surface as well as in the cytosol (Akira et al., 2001; Medzhitov, 2007). Their classifications include Toll-like receptors (TLRs) which are communicated on the cell surface or on the endosome's plasma layer, C -type lectin receptors (CLRs) and scavenger receptors which are just present on the cell surface (Figdor et al., 2002), and cytosolic PRRs such as NOD-like receptors (NLRs) and RIG1-like receptors (RLRs) which can aggregate to form inflammasomes (Bryan et al., 2010; Compan et al., 2015; Figdor et al., 2002). RLRs have a place with the RNA helicases family that explicitly distinguishes RNA species got from infections by viruses in the cytoplasm and facilitate anti-viral suppression through induction of type I interferons (Yoneyama & Fujita, 2008). NLRs establish a vast group of intracellular PRRs, for example, NOD1, NOD2 and NALP3 {NACHT

[neuronal apoptosis inhibitory protein (NAIP), CIITA, HET-E and TP-1}}, LRR (leucine-rich repeat) and PYD (pyrin domain containing protein 3)} (Martnon et al., 2009; Schroder & Tschopp, 2010; Ting et al., 2008). NOD1 and NOD2 bind to intracellular bacterial cell products, and NALP3 reacts to different stimuli to frame a multi-protein complex named the NALP3 inflammasome. Together, they initiate caspase-1 activation, which thus cleaves pro-IL-1 β , pro-IL-18, and pro-IL-33 and releasing their bioactive components (Fritz et al., 2006; Martnon et al., 2009). In addition, intracellular double stranded DNA (dsDNA) discharged by DNA viruses or bacteria work as PAMPs to initiate type I IFN through unidentified pathways (Ishii & Akira, 2006). In addition to PAMPs, endogenous molecules discharged by host cells due to damage, pathogen invasion, and harm, can also be recognized by TLRs, NLRs, RLRs (Shi et al., 2003). Molecules related with tissue damage, for example, uric acid, nucleotides, adenosine triphosphate (ATP), reactive oxygen species, and cytokines are discharged at the site of injection if tissue injury happens (Shi et al., 2003). Particulate vaccine adjuvants cause local tissue damage and cell death at the injection site hence can activate inflammmasomes (Kool et al., 2008). TLRs are the most studied PRRs in pigs but many other PRRs are not well understood. Because PRRs tend to share high sequence homology among species, synthetic/purified PAMPs used in murine or human studies are also presumed to stimulate innate immune receptors in swine. For instance, pigs respond to peptidoglycan (PGN) and lipoteichoic acid that stimulate TLR2, poly I:C stimulates TLR3 (Auray et al., 2010), lipopolysaccharide (LPS) stimulates TLR4 (Islam et al., 2012; Uddin et al., 2012), imiquimod (which belongs to a group of drugs called immune response modifiers) and ssRNA stimulate TLR7/8 (Calzada-Nova et al., 2010), CpG ODN stimulates TLR9 (Auray et al., 2010; Calzada-Nova et al., 2010; Dar et al., 2010) and muramyl dipeptide (MDP) (a constituent of both Gram-positive and Gram-negative bacteria) stimulates NOD receptors (Jozaki et al., 2009). This high degree of homology may facilitate research on the other PRR members.

However, NLRs, RLRs and CLRs have not been described in pigs.

Table 1.1 Pattern Recognition Receptors, localization, their ligands, and origin in humans, mice and pigs.

TLRs (Toll-like receptors) (present in mice, human and pig)	Localization	Ligands (PAMPs or DAMPs)	Origin of the ligands
TLR 1 (mice, human)	Plasma membrane	Triacyl lipoprotein	Bacteria (Takeuchi et al., 2002)
TLR 2 (mice, human and pig)	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self (Auray et al., 2010; Schröder et al., 2003)
TLR 3 (mice, human and pig)	Endolysosome	dsRNA	Virus (Auray et al., 2010; Sen & Sarkar, 2005)
TLR 4 (mice, human and pig)	Plasma membrane	LPS	Bacteria, viruses, self (Islam et al., 2012; Tapping et al., 2000; Uddin et al., 2012)
TLR 5 (mice, human)	Plasma membrane	Flagellin	Bacteria (Hayashi et al., 2001)
TLR 6 (mice, human)	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses (Henneke et al., 2005)
TLR 7 (mice, human and pig)	Endolysosome	ssRNA	Virus, bacteria, self (Calzada-Nova et al., 2010; Crozat & Beutler, 2004)
TLR 8 (mice, human and pig)	Endolysosome	ssRNA	Virus, bacteria, self (Calzada-Nova et al., 2010; Heil et al., 2004)
TLR 9 (mice, human and pig)	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self (Auray et al., 2010; Bauer et al., 2001; Calzada-Nova et al., 2010; Dar et al., 2010)
TLR 10 (only in human)	Endolysosome	Unknown	Unknown (Hasan et al., 2005)
TLR 11 (only in mice)	Plasma membrane	Profilin-like molecule	Protozoa (Yarovinsky et al., 2005)
TLR 12 and 13 only in mice	Endolysosome	Unknown	Unknown (Andrade et al., 2013)

1.1.1.2 Innate immunity in the skin

Before discussing innate immunity in the skin, a brief anatomy of the porcine skin will be provided.

1.1.1.2.1 Brief anatomy of porcine skin

The skin is the biggest organ of the body making up approximately 20% of the total body weight and it receives approximately 33% of all blood circulating through the body. The skin protects against environmental injuries such as biologic, physical, or chemical substances. Porcine skin has been broadly utilized as a substitute for human skin in different fields of dermatological research as they share certain similarities (Klíma et al., 2009; Laurent et al., 2007). Similar to human skin, porcine skin is partitioned into three histological layers from top to bottom namely: the epidermis, the dermis and the hypodermis (or subcutis) as described in figure 1, each with a particular role in the general skin functions (Laurent et al., 2007).

The most superficial layer of the skin is the epidermis, which is the first barrier of protection from the invading of foreign substances. The epidermis is composed of layers of flattened, anucleated cells and keratin filaments, interfilament matrix and glycolipid. A basal, cuboidal cell layer of epithelia settles upon thick basal lamina which protects the germinal cells that are important to the recovery of the layers of the epidermis. These germinal cells are isolated from the dermis by the basement membrane (Cartlidge, 2000). Epidermal turn-over is very high and is estimated to occur every 14-30 days based on the skin region.

The dermis is the thickest of three layers of skin and it contains elements necessary for thermoregulation and it supports the vascular system by supplying the avascular epidermis with supplements. The dermis contains fibroblasts, which emit collagen, elastin and ground substance that give the skin structural support and flexibility. Likewise present are immune cells that

protect against foreign antigens penetrating the epidermis. The hypodermis is the deepest layer of the skin comprised of a system of fat and collagen cells. It works as both a cover, preserving the body's warmth, and as a safeguard to the internal organs through shock absorption. It likewise stores fat as energy reserves for the body. The veins, nerves, lymph vessels, and hair follicles likewise cross through this layer (Cartlidge, 2000)

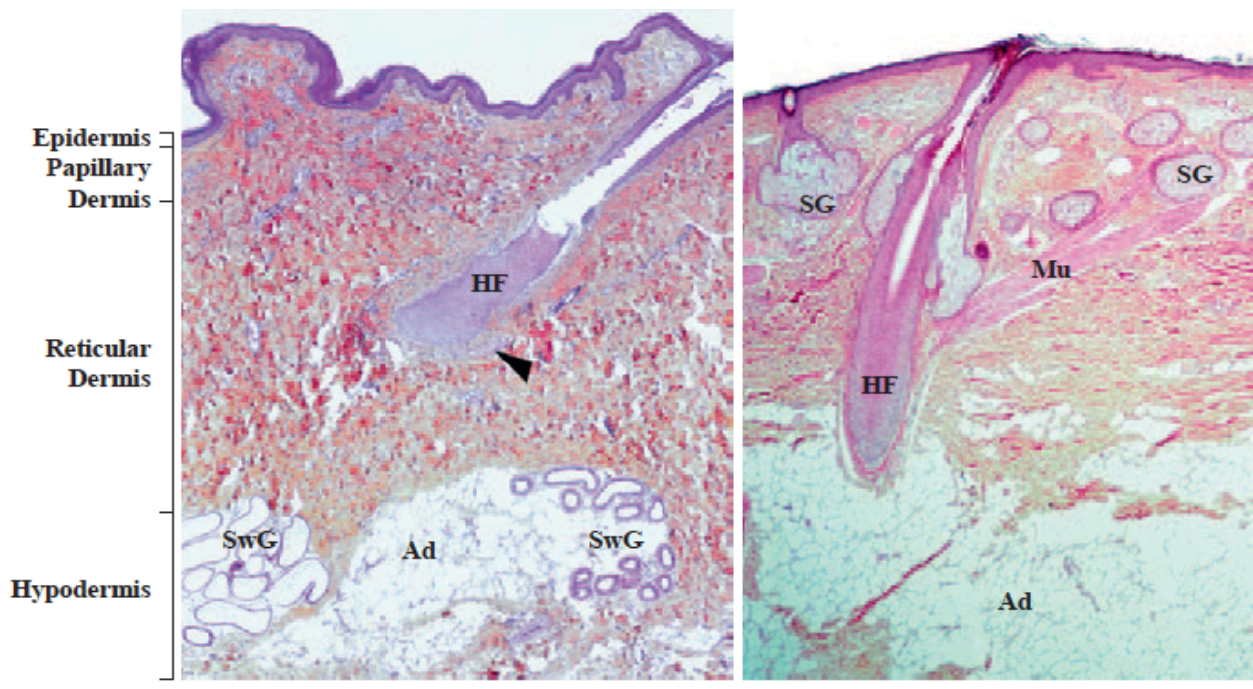


Figure 1.1 Comparative histological aspect of porcine (left) and human (right) skin (haematoxylin-eosin-saffron staining). HF: hair follicle, Mu and arrowhead: Arrector pili muscle, SwG: Sweat gland, SG: sebaceous gland, Ad: Adipocytes (hypodermis) adopted from (Debeer et al., 2013)

Skin innate immunity

The skin innate immune systems help to protect against pathogen colonization and invasion. The uppermost layer of the epidermis and the corneal layer in the eyes are unique layers not present in other epithelia that are exposed to the external environment (such as the gut and lung epithelia) (Kupper & Fuhlbrigge, 2004; Nestle et al., 2009). Dead keratinocytes in the epidermis and corneal layer provide the skin and eye with a physical barrier (Kupper & Fuhlbrigge, 2004; Nestle et al., 2009). Keratinocytes (sometimes referred to as "basal cells" or "basal keratinocytes") constitute 90% of the cells in the epidermis, the outermost layer of the skin (Baroni et al., 2012; Hsu, Li, & Fuchs, 2014). Keratinocytes produce antimicrobial peptides (AMPs) in response to infection including human β -defensins, cathelicidins and RNases (Otto,

2010; Pivaresi et al., 2004; Schaubert & Gallo, 2008) but the pig lacks α -defensins (Sang et al., 2009). DCs of the epidermis are known as Langerhans cells and they express PRRs which can detect invading microorganisms via PAMPs expressed on the invading microorganism cell surface; this interaction initiates early immune responses in the skin (Fournier & Philpott, 2005). The underlying dermis is anatomically more complicated, with greater cell diversity (Basset et al., 2003) including the granular, spinous and basal layers. The basal layer is primarily comprised of basal keratinocytes and undifferentiated cells, which can be viewed as the undeveloped cells of the epidermis. They develop into keratinocytes of the stratum spinosum, which then relocate externally. Other cell type also found inside the basal layer which incorporate melanocytes (pigment generating cells), Langerhans DCs, and Merkel cells (that have touch receptors) (Härle-Bachor & Boukamp, 1996; Jensen et al., 1999).

Immune cells present in the dermis include Langerhans DCs, macrophages, mast cells, B and T-cells, plasma cells, NK cells, fibroblasts and innate $\gamma\delta$ T-lymphocytes and invariant natural killer T-cells (iNKTcells) (Kupper & Fuhlbrigge, 2004; Nestle et al., 2009). These skin immune cells all express PRRs to detect invading pathogens. The skin is constantly exposed to external factors (e.g., ultraviolet radiation, pollution, topical medications, skin care products) that can alter the balanced relationship between the skin and its microbiome. Such disruptions may result in increased risk for infections, chronic inflammatory skin disease (e.g., atopic dermatitis, psoriasis, rosacea, and acne), pruritic, and irritated skin (Zeeuwen et al., 2013).

While an essential capacity of immune cells present in the skin is discovery of invading microorganisms through PRRs, another imperative capacity is to maintain the harmony between the host and the skin microbiome. It is thought that like commensal microorganisms found in the gastro-intestinal (GI) tract, skin microorganisms assume a crucial role in keeping pathogenic organisms from colonizing the skin's microenvironments. Bacteria are present on the skin

surface, the deeper layers of the epidermis, the dermis, and dermal adipose tissue (Weyrich et al., 2015). The majority of the microorganisms in the skin are harmless or beneficial, providing protection against pathogens modulating the host's cutaneous innate and adaptive immune systems (Baldwin et al., 2017). Commensal species of microorganisms that naturally reside on the surface of the skin are an integral part of the innate immune system. These bacteria protect against pathogen growth by competing for nutrients and space. Some bacteria directly restrict the growth of competitors via production of antimicrobial compounds that can inhibit reproduction of closely related species without affecting the organisms producing them.

Staphylococcus epidermis reside on normal skin and they have been shown to suppress inflammation by inducing secretion of the interleukin-10 by APCs (Chau et al., 2009; Lai et al., 2009). *Staphylococcus epidermis* also secretes a unique lipoteichoic acid that acts through a TLR2-dependent mechanism to inhibit inflammatory cytokine release from keratinocytes and induction of inflammation triggered by injury (Gallo & Nakatsuji, 2011; Lai et al., 2009). The balanced interactions between the host cells and bacterial populaces are influenced by host and environmental factors and this imbalance may contribute to chronic inflammatory skin diseases, such as atopic dermatitis, psoriasis, broacea, or acne (Rosenthal et al., 2011; Schommer & Gallo, 2013).

1.1.1.3.2 APCs activation on the skin and migration to the draining lymph node.

DCs (Langerhans cells and macrophages) present antigens to naive T cells. Epidermal Langerhans cells utilize their dendrites (arm-like projections) to survey the environment, particularly in the *stratum corneum*. Langerhans cells recognize PAMPs by PRR interaction then migrate to the lymph nodes draining the site of infection and present antigens to naive T-lymphocytes. DCs express explicit attachment molecules and develop chemoattractant receptors

that enable them to react to an assortment of ligands (Sozzani et al., 1997; Sozzani et al., 1995), which control their movement. For instance, to migrate towards and to invade non-lymphoid peripheral tissues, juvenile DCs (and a portion of their precursors, especially monocytes) use explicit chemokine receptor-ligand pathways, for example, CCR2-CCL2 (Geissmann et al., 2003; Merad et al., 2002), CCR5-CCL5 (Stumbles et al., 2001), and CCR6-CCL20 (Le Borgne et al., 2006). Once they take up antigen, DCs undergo a maturation process to become specialized APCs. Specifically, these mature DCs have reduced endocytic abilities, reduced expression of CCR5 and CCR6 and increased expression of CCR7, which helps to traffic to the draining lymph nodes and show expanded capacity to display and present antigen (Ohl et al., 2004; Willmann et al., 1998). Mature DCs have enhanced expression of MHC class II and co-stimulatory molecules (CD80, CD86 and CD40), development marker (CD83), CCR7 and CXCR4 (Lechmann et al., 2001; Ohl et al., 2004) and increased ability to secrete cytokines and chemokines. Antigen presentation and recognition by DCs plays a vital role in connecting innate and adaptive immune responses. Communication to T cells by DCs about the type of immune response that should be induced (i.e. Th1-type, Th2-type, etc.) is dependent upon the pathogen or antigen that has been presented, the local tissue condition at the site of infection and maturation signals.

Antigen presentation requires internalisation of the antigen, processing inside the cell, and display of a short peptide on the surface of the APC on an MHC molecule. There are two kinds of MHC: MHC class I and II. MHC class I is found on nucleated cells in the body and is utilized to present endogenous substances, for example, viral or tumor proteins to cytotoxic T cells. MHC class II is present on APCs (dendritic cells, monocytes/macrophages and B cells) and is utilized to present foreign non-self antigens to CD4⁺ T cells. Exogenous pathogens are endocytosed and displayed chiefly on MHC class II peptides on DCs. In the endoplasmic reticulum (ER) of DCs, recently formed MHC class II molecules are associated with preformed invariant chain (Ii, also

called CD74), which possesses the peptide-binding site (Lamb & Cresswell, 1992; Roche et al., 1991). It binds the MHC class II peptide-restricting notch with a locale in its luminal space that is called CLIP (for class-II-related invariant chain peptide). It is cleaved by numerous proteases in characterized successive cleavage steps, beginning at the carboxy-terminal luminal area, until just the CLIP part is left possessing the peptide-binding groove of MHC class II (Bryant & Ploegh, 2004). The cleavage of proteins and stacking onto MHC class II for presentation requires augmented acidification of endosomal compartments by the enactment of a proton pump within the mature DCs (Trombetta et al., 2003). The antigen-stacked MHC class II complexes are then transported to plasma membranes through tubular organelles (Kleijmeer et al., 2001; Savina & Amigorena, 2007).

Endogenous antigens (viral proteins, intracellular bacterial proteins, flawed cell products) and exogenous antigens that are processed inside the cell are only presented on MHC class I molecules (Savina & Amigorena, 2007). Intracellular proteins frequently have ubiquitin joined to them. These ubiquitin-conjugated proteins are broken down into short peptides of eight to ten amino acids by multifunctional proteases known as proteasomes. MHC class I proteins overlap and amass inside the ER lumen and peptide binding is a fundamental part of the assembly process. Peptides are translocated from the cytosol into the ER lumen, which is performed by the transporter associated with antigen processing (TAP) (Yewdell & Nicchitta, 2006). The gathering of this complex requires multiple chaperones, for example, calnexin, calreticulin, ERp57 and tapasin that help with collapsing and stacking of peptides on the MHC class I peptide-binding groove (Cresswell et al., 2005; Rock et al., 1990). After peptide stacking, MHC class I molecules separate from TAP and cluster at the export sites on the ER layer where they are specifically enlisted into cargo vesicles for transport to the Golgi apparatus. MHC class I molecules at that point traffic through the Golgi apparatus to the plasma membrane.

Upon antigen exposure and processing, DCs traffic to secondary lymphoid organs where they induce adaptive immune responses. The DC migration process starts with 1) recognition of mobilizing signals such as various inflammatory signals including PAMPs, DAMPs and pro-inflammatory cytokines such as TNF- α and IL-1 β . Pro-inflammatory cytokine exposure induces expression of CCR7 (Antonopoulos et al., 2001; Cumberbatch et al., 1999; Enk & Katz, 1992) which induces maturation of DCs which is manifest as rearrangement of their chemokine receptor expression, altered expression of adhesion molecules and eventually induced mobilization (Granucci et al., 1999). 2) Separation from the encompassing tissues where attachment molecules, for example, epithelial cadherin, maintain DCs in the peripheral tissues. Local TGF- β triggers an aberrant role in DC maintenance as it upregulates the expression of E-cadherin on DCs and represses its development and CCR7 expression (Geissmann et al., 1999). Pro-inflammatory cytokines TNF- α and IL-1 β instigate DC separation by diminishing E-cadherin expression (Jakob & Udey, 1998). 3) Interstitial relocation where DCs are segregated from tissue and move through the extracellular framework of proteins (collagens, fibronectins and laminins) and basement membranes before entering the lymphatics. To counter these obstructions, DCs up-regulate matrix metalloproteinases (MMP)-2 and MMP-9 (Ratzinger et al., 2002), and down-regulate tissue inhibitors of metalloproteinases (TIMPs) (Darmanin et al., 2007). DC passage into lymphatics is controlled by chemokine CCR7 and its ligands, CCL19 and CCL21 (Ohl et al., 2004) and also CXCR4 and its ligand CXCL12 (Kabashima et al., 2007). Secretion of TNF- α further enhances CCL21 expression by lymphatic endothelial cells, which guides DCs towards lymph vessels (Martín-Fontecha et al., 2003). 4) DC migration through afferent lymphatic endothelium is a steady state migration guided by a chemokine scavenging receptor, D6 and CCX-CKR1 expressed on the lymphatic endothelium (Heinzel et al., 2007; Mantovani et al., 2006). 5) DCs travel through the afferent lymphatics into lymph nodes which are bolstered by the

intercellular adhesion molecules (ICAM)-1 and vascular cell adhesion (VCAM)-1 on the lymphatic endothelium (Johnson et al., 2006). 6) When DCs undergo maturation following activation, they downregulate their responsiveness to inflammatory chemokine pathways and migrate to the draining LNs by upregulating CCR7, which responds to CCL19 and CCL21 expressed by the lymphatic vessels draining the site of infection (Dieu et al., 1998; Sallusto et al., 1998; Sozzani et al., 1998). DC movement in lymph node is guided by CCR7-CCL19/CCL21 while CCR8 and its ligand CCL1 control DC movement in the lymph node parenchyma (Qu et al., 2004). DCs also have high-affinity for CCL19 and CCL21, which are uniquely involved in DCs homing to T cell zones of lymphoid tissues. The main source of CCL19 and CCL21 is fibroblast reticular cells within the T cell zone. Additionally, naive T cells express CCR7, and this is the reason naive T cells circulate through similar areas of lymph nodes where antigen-bearing DCs are concentrated. The co-localization of antigen bearing activated DCs and naive T cells augment the opportunity of T cells with receptors for the antigen finding that antigen.

Follicular dendritic cells (FDCs) are conspicuous stromal cell constituents of B cell follicles with the capacity to hold complement fixed antigens on their cell surface for expanded time frames (McCloskey et al., 2011). FDCs secrete the B cell attracting chemokine CXCL13 (McCloskey et al., 2011). Germinal centre (GC) B cells express the CXCL13 binding chemokine receptor CXCR5 and are thereby attracted towards the B cell follicle (McCloskey et al., 2011). FDCs produce B-cell activating factor (BAFF), which is involved in regulating GC B cell survival (Borhis et al., 2017). FDCs present antigen to GC B cells where antigen-exposed GC B cells perceive the antigen through their BCR, endocytose and process it into peptides then present to T follicular helper cells (T_{FH} cells) in the type of peptide-MHC II (Zhang et al., 2005). T_{FH} cells at that point supply related B cells with survival signals. It is expected that after each round

of somatic hypermutation, B cells with high-affinity BCRs can recognise antigen introduced by FDCs and, subsequently can interact with T_{FH} cells. This prompts the positive selection of protective T and B cells, while others bearing lower affinity receptors cannot compete for limited antigens and hence undergoes apoptosis. To avoid autoimmunity, these cells must be cleared effectively. FDCs discharge the apoptotic cell binding protein Mfge8 that are then recognized and cleared by macrophages.

The innate immune system can be activated through ligand binding to a number of conserved PRRs, such as the TLRs (Underhill & Ozinsky, 2002), NLRs, RLRs and CLR (Stahl & Ezekowitz, 1998) which can be stimulated by many adjuvants. Targeting the host innate immune system through activation of immune receptors constitutes an important approach in the development of immunostimulatory adjuvants. Hence, adjuvants that stimulate PRRs may enhance the protective immune response and promote memory to a specific antigen (Cox & Coulter, 1997).

1.1.2 Overview of adaptive immunity

Adaptive (acquired) immunity refers to antigen-specific defense mechanisms that react to and eliminate a specific antigen and is designed to take several days to become fully activated (Abbas et al., 2014a). There are two noteworthy parts of the adaptive immune responses: humoral and cell-mediated immunity. Humoral immunity is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins, and certain antimicrobial peptides. Cell-mediated immunity is mediated by T lymphocytes and involves the activation of cytotoxic T lymphocytes and Th1-type T or Th2-type T cells that promote macrophages and NK cell activation (Abbas et al., 2014a; Itano et al., 2003).

Substances that are targeted by antibody molecules and antigen receptors on lymphocytes are called antigens while an immunogen is an antigen that is recognized by the body as non-self and activates an adaptive immune response (Abbas et al., 2014a). During adaptive immunity, antigens are taken up by APCs then transported to lymphoid organs where they are recognized by naïve B-lymphocytes and T-lymphocytes. The activated B and T lymphocytes then proliferate and differentiate into effector cells.

1.1.2.1 Naïve T and B cell activation in draining lymph nodes

T cells are unable to recognise pathogens directly (Mackay et al., 2012). Generation of activated effective T cell requires three vital signals. Recognition of antigen displayed on MHC molecules through TCR on T cells is the first signal. The receptor on the surface of a $CD4^+$ T cell binds to the peptide presented on MHC class II complex on the surface of APCs while $CD8^+$ T cell binds to the peptide presented on MHC I complex on the surface of the APCs (Banchereau & Steinman, 1998). Effective antigen presentation leads to maturation of naïve T cells to effector T cells, which then differentiate into two cytotoxic $CD8^+$ T cells and $CD4^+$ T cells. The state of activation of DCs and the presentation of antigen on MHC I or II are critical factors in determining the outcome of T cell responses (Mellman & Steinman, 2001). Additionally, immunological synapse formed by prolonged and dynamic interaction of DCs and T cells is necessary for effective activation of T cell. At first, ICAM-1 found on DCs forms a limited interaction with leukocyte function associated antigen 1 (LFA-1) on the T cells (Dustin et al., 2006). The co-stimulatory molecule CD80/CD86 form a ligation with CD28 on T cells provides the second signal, which further stabilises the interaction (Lotze & Thomson, 2001). Furthermore, cooperation between CD40 on the T cells and CD40L on the DCs activates DCs to enhance up-regulation of co-stimulatory molecules that aids in stabilizing the immunological

synapse (Lanzavecchia & Sallusto, 2001). DCs release cytokines to provide the third signal which then determines the differentiation status of the T cells.

Two distinct signals are required for B cell activation and differentiation into memory B cells or plasma cells. The first signal of activation occurs when antigen binds to BCRs. After binding to the BCR, the antigen is taken up by receptor-mediated endocytosis, broken down into small peptides, and incorporated into MHC class II molecules on the surface of B cells (Batista et al., 2001). Introduction of an antigen on MHC II molecule on a B cell empowers the B cell to presents antigen to T cells (Foy et al., 1994; Lederman et al., 1992).

The second activation signal occurs via either a thymus-dependent or a thymus-independent mechanism (Lederman et al., 1992). T helper cells, which are mainly follicular T helper (T_{FH}) cells, initially activated with similar antigen recognize and bind MHC-II-peptide complexes via T cell receptor. T cells express the surface protein CD40L which acts as an essential co-stimulatory factor for B cell activation (Dubois et al., 1997). CD40L binding to CD40 promotes B cell proliferation, immunoglobulin class switching and somatic hypermutation as well as helps sustain T cell growth and differentiation. This activates the BCR to form microclusters and trigger downstream signalling cascades (Depoil et al., 2008). The microcluster undergoes a contraction phase to form an immunological synapse which helps in the stable interaction between B and Th2-type T cells to give a bidirectional activation signals (Depoil et al., 2008; Fleire et al., 2006).

To produce antigen-specific antibodies, B cells require cytokine signalling and stimulatory signals from Th T cells in the lymph node (Kaliński et al., 1999). Th2-type T cells produce IL-4 following their recognition of specific ligand on the surface of the B-cell, which interact with CD40L and synergizes the clonal expansion that precedes antibody production (Dubois et al., 1997). IL-4 secreted by the Th2-type T cells is directed at its site of contact with

the B cell hence acts specifically on the antigen-specific target B cell (Andrew et al., 1998). The combination of B-cell receptor and ligation of CD40 along side with IL-4 in addition to MHC and T-cell receptor derived from direct T-cell contact prompts the B-cell proliferation (Rathmell et al., 1996).

In contrast, some antigens that can directly give the second B cell activation signal (thymus-independent activation). These antigens may include components of some bacterial cell wall such as lipopolysaccharide or antigens containing exceedingly repetitious molecules such as bacterial flagellin (Edwards et al., 2002; Wortis et al., 1995). Upon activation, B cells proliferate and form germinal centers where they differentiate into memory B cells or plasma cells (Foy et al., 1994; Paus et al., 2006). Expansion of B cells leads to the generation of a specific antibody such as IgG, IgA or IgE, depending on the nature of the antigen (Batista & Harwood, 2009). This process leads to the generation of memory B cells and long-lived plasma cells that confers long-lasting immunity from infection (McHeyzer-Williams et al., 2012; Penna et al., 1996). Following differentiation into plasma cells, additional signals initiate plasma cell antibody class switching and regulate antibody secretion (Reimold et al., 2001).

CD4⁺ cells can differentiate into two kinds of memory cell with very different characteristics of activation. One sort of these cells are called effector memory cells since they can quickly develop into effector CD4⁺ T cells and discharge copious amount of IFN- γ , IL-2, IL-4, and IL-5 early after restimulation. The effector memory cells lack the chemokine receptor CCR7 though they express β -1 and β -2 integrins in high levels and inflammatory chemokines receptors suggesting that effector memory cells are highly specialized for quickly entering inflamed tissues. The other kinds of memory cells are called central memory cells and they express CCR7 that allows them to recirculate more easily to secondary lymphoid tissues T cell zones. These central memory cells are highly sensitive to cross-linking by T-cell receptor and

rapidly upregulate CD40L in response to it. Be that as it may, they take more time to separate into effector cells and in this way do not discharge as much cytokine as do effector memory cells early after restimulation. Activated Th2-type T cells trigger B cell stimulation and production of antibodies. Th2-type T cells secrete IL-4, IL-5, IL-6 and IL-10 cytokines. They likewise initiate activation of eosinophils (Mosmann & Sad, 1996). Th2 cells are associated with atopic dermatitis in the skin. Th17-type T cells secrete IL-17 and IL-22 which are important in protection from bacterial and parasitic infections in the skin (Louten et al., 2009).

Th cells can be Th1, Th2, and Th17-type T cells. Each has explicit signalling cytokines and effector capacities (Walsh & Mills, 2013). Th1-type T cells are involved in a cell-mediated immune responses that function to kill intracellular pathogens through production of IFN- γ which activate macrophages and NK cells (Biswas & Mantovani, 2010). Th1-type T cells lead to increased cell-mediated responses, Th1-type T effector cells utilize two signs to initiate macrophages activation. They specifically produce IFN- γ , which binds to IFN- γ receptors on the surface of macrophage, and also display the costimulatory protein CD40 ligand, which binds to CD40 molecule on the macrophage. When activated, the macrophage can kill the phagocytosed organisms by allowing the lysosomes fuse more promptly with the phagosomes, releasing a hydrolytic attack, and also the activated macrophages makes oxygen radicals and nitric oxide, the two of which are very harmful to the organisms. Because DCs likewise express CD40, the Th1-type T cells at locales of infection can likewise aid in their activation. Thus, the dendritic cells increase expression of MHC class II molecules, B7 (CD80/86) costimulatory proteins, and different cytokines, particularly IL-12. This makes them increasingly successful in stimulation and development of Th1-type T effector cells in peripheral lymphoid organs by providing a positive feedback loop that leads to the generation of Th1-type T cells, which then activate the macrophages.

Other T cell populations, for example, regulatory T cells (Tregs), modify the immune cell's response to foreign and self-antigens thus preventing autoimmune diseases. Several suppression mechanisms of Treg include local secretion of immunosuppressive cytokines such as IL-10 and TGF- β , cell-contact-dependent suppression, and modification of function or killing of APCs. For example, depletion of Treg-secreting IL-10 and TGF- β contribute to inflammatory bowel disease in mice (Read, Malmström, & Powrie, 2000). Tregs coexpressing Foxp3 and IL-10 at a solitary-cell level are found in *lamina propria* of the intestine and blood but not in the spleen (Maynard et al., 2007; Uhlig et al., 2006). TGF- β may mediate direct suppression of the immune responses by conditioning responder T cells to be sensitive to suppression, maintaining Foxp3 expression and increasing Tregs suppressive activity. Foxp3⁺ characteristic Tregs produce immunosuppressive IL-35, a recently recognized member from the IL-12 family (Collison et al., 2007).

Alternatively, assimilation of cytokines by Tregs may cause apoptosis in responder T cells (Pandiyani et al., 2007). Granzyme or perforin-dependent cell-to-cell mechanism by Tregs, or delivery of a negative signal to responder T cells might also kill responding T cells or APCs. Some of these negative signals include upregulation of intracellular cyclic AMP, which impedes T cell proliferation and secretion of IL-2, or Treg-expressed generation of pericellular adenosine catalyzed by CD39 (ectonucleoside triphosphate diphosphohydrolase1) and CD73 (ecto-5'-nucleotidase). Activated Tregs may likewise down regulate expression of CD80/86 on APC or modulate dendritic cells to form the enzyme indoleamine 2, 3-dioxygenase that catabolizes the essential amino acid tryptophan to kynurenines which are then toxic to T cells; both of these mechanisms are reliant on the expression of CTLA-4 by Tregs.

Cytotoxic CD8⁺ T cells recognise and bind to MHC class I molecules on all nucleated cell types, which present antigen. They bind to the Fas death receptor, a protein on the cell

membrane on the surface of the target cell thus starts the perforin–granzyme pathway and cytokine-mediated pathways to initiate apoptosis, which specifically executes virally infected cells or tumour cells (Cao et al., 2007; Rouvier et al., 1993).

1.1.2.1.1 Function of activated lymphocytes

Clearance of intracellular pathogens and tumours cells depends on the cell-mediated immune response. CD8⁺ effector T cells play an important role with their function distinguished by antigen-specific cytotoxicity restricted by MHC class I and the killing is Fas-Fas perforin mediated. Upon recognizing a cell that is presenting its cognate antigen on MHC class I, CD8⁺ T cells secrete perforin and granzymes, which are cytotoxic proteins at the point of contact with the target cell leading to killing of specific cell. Perforin is a membrane-disrupting protein, which increases apoptosis induced by granzymes on the target cell. The cytotoxic synapse of CTL often referred to as a ‘the kiss of death’ because of its ability to cause death of the target cell. There are two described mechanisms of CTL-mediated killing which are 1) Ca²⁺-dependent killing by perforin and granzymes, and 2) Ca²⁺-independent killing mediated by binding of Fas ligand (FasL) to Fas (CD95) on target cells (Dustin et al., 2006; Fooksman et al., 2010). Apoptosis triggers death in both though the perforin pathway is more expedient. Perforin-mediated killing is progressively broad and depends on exceedingly conserved membrane responses to injury and lysis of endosomal membranes leading to introduction of granzymes into the cytoplasm of the target cell with consequently no requirement of a particular receptor (Dustin et al., 2006; Fooksman et al., 2010). Since perforin and granzymes are released from the cell, the formed synaptic cleft improves the function via high local concentration and further preventing exposure to bystander active perforin in the vicinity. Fas, likewise, requires cell-cell contact, since FasL

and Fas are proteins membranes with a restricted reach (~15 nm). FasL present in the granules of CTLs that co-express perforin and granzymes to be exocytosed into the synaptic cleft because of TCR activation (Fooksman et al., 2010). In addition to cytotoxicity, CD8⁺ effector cells produce IFN- γ and TNF- α that activate macrophages to kill the infected cell (Intlekofer et al., 2005).

CD4⁺ T-cell priming ends up in different subsets recognized by the generation of specific cytokines and effector capacities. Traditionally, CD4⁺ effectors were classified as Th1/Th2-type T cells however different subsets exist including IL-17 secreting T cells (Th17-type T cells) and Tregs have since been characterised (Weaver et al., 2006).

Th2-type T cells secrete IL-4, IL-5 and IL-13 that are necessary for B-cell antibody production (note that IL-4 is not expressed by pigs). These cytokines drive B-cell multiplication by secretion of IL-4 and contact-dependent CD40 ligand on DCs and CD40 on the T-cells binding, which augments humoral guards against extracellular pathogens. Besides, these cytokines empower IgE generation and eosinophilic inflammation, imperative for the protection against helminthic infection, also profoundly pertinent to the allergic immune responses (Soumelis et al., 2002).

Th1-type T cells are necessary for activation of macrophages through IFN- γ secretion and also contact-dependent stimulation by utilizing an assortment of cell surface costimulatory ligands. Th1-type helper T cells hence assume a crucial role in clearance of intracellular pathogen, delayed type hypersensitivity reactions and down regulation of B-cell responses (Jankovic et al., 2007). Th1-type T cells differentiation is directed by IFNs generated by the innate response to infection.

Th17-type T cells secrete IL-17, IL-17F, IL-6, and TNF, and are currently viewed as a particular CD4⁺ T-cell subset in mice, human, cattle and canines. IL-17 is a powerful inflammatory cytokine associated with the proliferation and recruitment of neutrophils. Th17-

type T cells differentiation and survival requires TGF- β , IL-6, and IL-23 (Bettelli et al., 2007). CD4⁺ T cells can likewise differentiate into cells with the capacity for suppression of T cell responses which prevent autoimmunity commonly referred to as Tregs (Sakaguchi, 2005).

Memory T cells are categorized into 2 subsets termed central memory (TCM) and effector memory. TCM cells express CD45RO, CD62L, and CCR7, and are sequestered in lymphoid tissue (Broli, 2013; Sallusto et al., 2004). They react to antigen by undergoing rapid division to differentiate into effector cells; however all by themselves without the antigen they have almost no effector function. TEM cells express CD45RO but neither CD62L nor CCR7 (Sallusto et al., 2004), circulate in the peripheral tissues and have limited proliferative ability but increasingly more effector functions. In this way, TEM cells performs surveillance of the peripheral tissues for fast effector responses, though TCM cells rapidly create reinforcement effector cells. At the point when the immune responses turn out to be increasingly localized, the immune synapse establishes a lineage toward effectors, and the daughter cell distal establishes a lineage toward TEM (Klebanoff et al., 2005; Sallusto et al., 1999). B-cells once activated by antigen in secondary lymphoid tissues, generate memory B cells and antibody-secreting plasma cells (Odendahl et al., 2005).

1.1.2.2 Humoral immunity in the skin

B memory cells are effector cells that have already been exposed to antigens to guarantee a quicker and long-lasting immune response. B cells secrete specific antibodies (immunoglobulins) that can bind to explicit antigens (Martin & Kearney, 2000). Immune response effector capacities induced by antibodies are 1) Neutralization where antibodies bind to the pathogen and counteract adherence to the target cells, 2) Opsonisation wherein antibodies coat the antigen or microorganism surface and increase the consequent take-up by phagocytic

cells (Burton, 2002), 3) Activation of complement which leads to cleavage of C3 by enzyme C3 convertase to generate C3b. Next, C3b advances cleavage of C5 convertase to deliver C5b which is trailed by the commencement of the terminal common complex (Shishido et al., 2012). The whole process results in the membrane attack complex (MAC) formation, which causes lysis of the host or pathogenic cells by the development of pores in the cell membrane. C3b opsonisation enhances the recognition of pathogens by complement receptor 3 (CR3) expressed on the surface of neutrophils and macrophages. Cleavage products recognized by complement receptor 2 (CR2) on the B cell triggers production of antibodies (Molina et al., 1996). CR2 is one of several receptors for human CD23 (Aubry et al., 1994; Aubry et al., 1992). CD23 is an immunoregulatory protein discovered both on cell membrane and as a soluble protein. CD23 communicates with CR2 to enhance production of IgE with the help of IL-4 (Aubry et al., 1992), rescue germinal-center B cells from apoptosis (Bonney et al., 1993), provide T cell-activating signals by B cell APCs (Grosjean et al., 1994), and promote T-B cell adhesion (Björck et al., 1993). The C3a and C5a released from their respective convertases are potent mediator of anaphylatoxin reactions.

1.1.2.3 Cell mediated immunity (CMI) in the skin

The skin contains resident T cells and recruited circulating T cells. The skin is home to approximately 20 billion T cells, about double the number present in the whole blood volume (Clark et al., 2006). CD8⁺ T cells that are found in the epidermal region of the skin are of memory phenotype (Gebhardt et al., 2009) and live together with keratinocytes with particular localization close to LCs (Foster et al., 1990). Equal numbers of CD4⁺ and CD8⁺ cells are restricted to capillaries and the epidermal–dermal junction which are characteristic for the dermis (Mueller et al., 2013; Nomura et al., 2014). Large portions of the lymphocyte population are memory cells

communicating cutaneous lymphocyte-related antigen. Skin memory T cells hold key position and make the primary line of protection against pathogen challenge (Schenkel & Masopust, 2014). The Th17, Th1 and Th2-type T cells are important effector cells in inflammatory skin pathology such as allergic inflammation (Conrad et al., 2007; Honda et al., 2013) or psoriasis (Conrad et al., 2007). IL-17 together with IL-22 produced by Th17-type T cells induces abnormal differentiation of keratinocytes in psoriasis but Th17-type T cells shield skin from bacteria and fungal infections, for example, *Candida albino*s, *Klebsiella pneumonia*, and *Staphylococcus aureus* (Kashem et al., 2015; Kurebayashi et al., 2013). The extent of immune responses in skin is effectively controlled by Tregs which constitutes 5– 10% of all inhabitant skin T cells (Clark et al., 2006). Together with other resident skin T cells, Tregs effectively move between the skin and lymph nodes amid immune response as well as in the steady state (Clark, 2010; Tomura et al., 2010). In skin, Tregs regulate responses of T cells, APCs such as DCs and macrophages, as well as neutrophil accumulation during early stages of inflammation (Richards et al., 2010; Tiemessen et al., 2007). Tregs induces anti-inflammatory functional profile in macrophages and inhibit macrophage TNF- α production in the skin (Tiemessen et al., 2007)

The goal of vaccination is induction of protective immune memory that is long lasting and can respond rapidly to future infections. This is accomplished by exploiting the adaptive portion of the immune system usually characterized by durability and specific recognition of pathogens. Be that as it may, the magnitude, quality and duration of adaptive responses are exceedingly impacted by the innate portion of the immune system, which is characterized by limited specificity and immune memory. Activating the innate receptors tweaks the “immunogenic conditions” in which APCs and CD4⁺ T cells engrave the adaptive responses

1.2 Conventional vaccines

All vaccines contain an active component (the antigen), which is the target of the (hopefully) protective immune response. The main types of vaccines can be classified according to the following: attenuated (live), inactivated, toxoid, subunit vaccines, conjugate, DNA and live vectored vaccines.

1.2.1 Live, and/or attenuated vaccines

Live, attenuated vaccines are comprised of microbes that are living but have been passaged in culture to reduce replicating ability and pathogenicity (Meeusen et al., 2007). Being very similar to the microbe that causes natural infection, these vaccines are generally highly immunogenic and may not require addition of adjuvants (with some exceptions) to induce protective immune responses (da Costa et al., 2015; Makoschey, 2012; Rizzi et al., 2012). These vaccines tend to elicit strong cellular and antibody responses and often confer lifelong immunity with only one or two doses. These classes of vaccines have been very successful. Measles, mumps, and rubella (MMR) vaccine is a good example of attenuated live viral vaccine which has been used in the United States since 1971 (Ravanfar et al., 2009). Priorix ® is an MMR vaccine marketed by GlaxoSmithKline and contains these attenuated virus strains, measles (the Schwarz strain), mumps (the RIT 4385 strain), and rubella (the Wistar RA 27/3 strain) which are independently obtained by chick embryo tissue cultures of mumps and measles or MRC5 human diploid cells in the case of rubella (Wellington & Goa, 2003). Other examples of live, attenuated vaccines include smallpox, polio, yellow fever, tuberculosis, typhoid, anthrax, varicella (chickenpox), rotavirus and influenza vaccines (Flushield ®). For example, the virtual eradication of rinderpest virus from the globe is widely believed to have been critically dependent on the use of the “Plowright” vaccine. This is an attenuated vaccine produced from the Kabete O strain

passaged 90 times in tissue culture. However, the attenuated microbe have a small probability of reverting to a virulent form and cause infections, which may be particularly harmful to individuals who have damaged or weakened immune systems (immunocompromised hosts) such as those infected with HIV/AIDs, children and the elderly

1.2.2 Inactivated vaccines

Inactivated vaccines are produced by inactivating the microbe with chemicals, heat, or radiation (Meeusen et al., 2007). The fundamental characteristic of killed or inactivated vaccines over live attenuated vaccines is that they are safe. Since the pathogens are killed/inactivated the possibilities of reverting back to a virulence form to cause infections are obviated. However, this forms a gigantic drawback since the absence of replication results in quick clearance from the body prompting a diminished immunogenicity compared to the live vaccines. Killed/inactivated vaccines in comparison to subunit vaccines give rise to a more complex or greater inflammatory immune response since high amount pathogenic components and possibly some tertiary structures are preserved. Inactivated vaccines are widely used because they are not likely to revert to virulence in the non-immunocompromised and are therefore considered safe in addition to low doses of antigen. An example of such a vaccine is the Hepatitis A vaccine Epaxal® from Crucell where the strain RG-SB of hepatitis A virus is formalin inactivated and adsorbed onto a virosome formulation containing the adjuvant system (Bovier, 2008). Other examples of killed/inactivated vaccines have been developed for Typhoid, cholera, polio and rabies.. For example, a one-dose inactivated porcine circovirus type 2 (PCV2) vaccine has recently been licensed in the United States for the prevention of postweaning multisystemic wasting syndrome in pigs. However they are not as immunogenic as live, attenuated vaccines and they often require the addition of adjuvants and/or require two or more doses to induce protective immune response (Makoschey,

2012).

1.2.3 Convectional subunit Vaccines

Subunit vaccines, like inactivated whole-cell vaccines, do not contain live components of the pathogen. Instead of the entire microbe, subunit vaccines only include the antigen components of the microbe that best stimulate a protective immune system (Dintzis, 1992). By selecting only one or a few antigens as targets, these vaccines are must be extensively tested to ensure that the particular combinations of antigens and adjuvants generate an effective immune response. Like inactivated vaccines, subunit vaccines do not contain live components and are considered very safe. *Bordetella pertussis* vaccine is a good example of a subunit vaccine and contains inactivated *Bordetella pertussis* toxin (protein) with at least one other bacterial components. The pertussis toxin is detoxified by chemical treatment or by utilising molecular genetic techniques which may impact recognition by the immune system. In other examples, subunit vaccines use epitopes that are very precise regions of a protein that are recognised by T cells. A recombinant baculovirus producing the protective ORF2 protein of PCV2 has recently become available as a vaccine for pigs. These peptides are poorly immunogenic thus require addition of adjuvants to induce protective immune responses (Reed et al., 2009). Examples of subunit vaccines are hepatitis B and pertussis (whooping cough).

1.2.4 Toxoid Vaccines

The immune system produces antibodies that bind to and can potentially block bacterial toxins. Toxoid vaccines are made from toxins (poisons) that are produced by specific organisms (e.g. tetanus or diphtheria) that attack the circulatory system and are, to a great extent, responsible for manifestation of the disease. The toxin is rendered harmless by formalin fixation or another

means then included as an antigen in the vaccine to evoke an immune response. To increase the immunogenicity, the toxoid can be adsorbed to adjuvants. Toxoid vaccines are often considered safe because they do not have the ability to revert to virulent form that can cause. Further, toxoids tend to be stable, as they are less susceptible to changes in temperature, humidity and light (Baxter, 2007). However, because they are poorly immunogenic, they require addition of adjuvants to induce protective immune responses (Arimitsu et al., 2004). Vaccines against diphtheria and tetanus are examples of toxoid vaccines.

1.2.5 Conjugate Vaccines

Some components of the organisms e.g polysaccharides that coat bacteria can disguise or hide surface antigens so that they escape recognition by the immune system. Conjugate vaccines, a special type of subunit vaccine, overcome this problem (Dintzis, 1992). In comparison to plain polysaccharide vaccines, the polysaccharide is bound to a carrier protein to enhance their immunogenicity. When incorporated in the vaccine, the linked polysaccharide-carrier protein helps the immature immune system react to polysaccharide coatings and defend against the disease-causing bacteria. Conjugate vaccines also create a response against the molecules in the pathogen's capsule. Different protein transporters are utilized for conjugation, including diphtheria and tetanus toxoid. Conjugate vaccines have not been related with any uncommon, progressively extreme immune responses but continued surveillance for possible unexpected effects as with the introduction of any new vaccine is necessary. A vaccine that protects against *Haemophilus influenzae* type B (Hib) is a conjugate vaccine. However, they are poorly immunogenic thus require addition of adjuvants to induce protective immune responses but the benefits of using conjugates type of vaccines would depend on the antigen and adjuvant used, which may limit the scope of the application.

1.2.6 DNA Vaccines

DNA vaccines are comprised of genes that code antigens. DNA vaccines are an attractive class of new generation of vaccines because of their simplicity and for the many benefits they have over conventional vaccines. The fundamental principle behind DNA vaccines is that plasmid DNA (pDNA) encodes the antigen which transduces host cells instead of infusing antigen as peptide or protein (Bins et al., 2013). Upon DNA vaccination, host cells produce the protein (antigen) encoded by the DNA and immunity against this particular protein is subsequently induced. The major benefits related with DNA vaccines are that they are relatively inexpensive to produce, and they can evoke both humoral and cellular immune responses (Bins et al., 2013). In addition, pDNA is fairly stable at room temperature hence they do not require cold chain storage.

A DNA vaccine can elicit a strong antibody response to soluble antigens in addition to stimulating a strong cellular response to the microbial antigens present on surfaces of the cell. Poor immunogenicity remains the single biggest obstacle to human DNA vaccines achieving their potential. Adjuvants or delivery methods are required to enhance the immunogenicity of DNA vaccines (Meeusen et al., 2007). DNA vaccines targeting influenza and herpes viruses are being tested in clinical trials.

1.2.7 Recombinant Vector Vaccines

Recombinant vector vaccines are exploratory vaccines like DNA vaccines, yet they utilize an attenuated microbe to introduce and/or carry microbial DNA into cells. (Rizzi et al., 2012).

Recombinant vector vaccines have a few attributes that make them proficient in initiating humoral and cell-mediated immune responses, including their capacity to infect cells and persist in the body. They can infect APCs, and the viruses' proteins can act as adjuvants. . Recombinant

vector vaccines such as adenoviruses (Ads) can be attenuated to reduce pathogenicity, such as replication-deficient mutants. They can be administered by the oral or nasal route to promote immunity on the mucosal surfaces (Abbink et al., 2007). Contrasted with other viral delivery systems, Ads have limited cloning capacity and human Ads have a confined range of hosts which frequently makes testing in animal models troublesome (Abbink et al., 2007). Various organisms have been utilized for vector vaccines including adenoviruses, attenuated poliovirus and vaccinia virus. Meanwhile, a subunit vaccine capable of preventing Newcastle disease virus (NDV) in poultry was successfully registered. Hemagglutinin-neuraminidase (HN) protein, a protective antigen, of NDV was produced in plant cells and demonstrated to protect a vaccinated chicken once challenged with wild type virus. These vaccines are highly immunogenic and do not necessarily require addition of adjuvants to induce protective immune responses as the carrier vector used for delivery of the vaccine may as well act as an adjuvant.

1.3 History of vaccine adjuvants

The overall goal of vaccination is to generate strong immune responses to the administered antigen so that any future encounter with the antigen (likely as part of an invading pathogen) will trigger a protective memory immune response. Unlike natural infections or in response to vaccines comprised of live-attenuated pathogens, subunit vaccines or vaccines comprised of killed pathogens often require the addition of immune enhancing additives commonly known as adjuvant (Bomford, 1998). The word adjuvant comes from the Latin word *adjuvare* meaning “to help” or “to enhance” (Brown, 1998). Hence, adjuvants are broadly defined as compounds that enhance the specific immune response against co-inoculated antigens. Most of the initial studies that have contributed to the advancement of adjuvants for use in vaccines incorporated antigen and adjuvants both derived from the bacteria (Ramon, 1925, 1926; Ramon, 1940). The first

documented study with a substance that acted as a depot for the antigen at the site of delivery was lanolin and this adjuvant was administered with a killed salmonella bacterial vaccine in 1916 (Kensil et al., 2004).

There are four recognised periods for development of adjuvants including: a period for studying adjuvants for toxoid vaccines (1920s-1940s), utilization of oils and aluminum compounds as adjuvants (1940s-1970s), a period for synthetic adjuvants development and second-generation delivery depot systems (1970s-1990s), and development of receptor-related adjuvants that stimulate innate immune system (1990s-Present) (Petrovsky & Aguilar, 2004).

1.3.1 Development of adjuvants for toxoid vaccines

The concept of adjuvants in vaccines advanced significantly in the 1920s with Ramon et al. taking note that horses that had a boil at the vaccination site of diphtheria toxoid produced higher antibody titres (Ramon, 1925, 1926). It was later discovered that abscesses created by the addition of substances (adjuvants) alongside the diphtheria toxoid generated robust immune responses against the toxoid (Ramon, 1925, 1926).

The first vaccine adjuvanted with alum was made by co-precipitation of diphtheria toxoid with aluminum dissolved in carbonate buffer (pH 8.0), resulting in a co-precipitate of aluminum hydroxide and diphtheria toxoid (Glenny, 1926; Glenny & Barr, 1931). Inclusion of aluminium salts in subcutaneous injection triggered more rapid induction of humoral immunity and significant anti-toxoid antibody without local reactions when studied in guinea pigs (Ramon, 1925, 1926) and these salts were generally, but not correctly, referred to as alums (Glenny, 1926; Glenny & Barr, 1931). Since then aluminum salts, such as aluminum hydroxide, aluminum phosphate, and aluminum potassium sulfate have been used safely in vaccines as adjuvants due to their ability to strengthen the body's immune response to these vaccines.

1.3.2 Use of oils and aluminum adjuvants

As observed by Freund: “Interest in promoting antibody formation by addition of unrelated substances to antigens has never been lacking” (Freund, 1947). In 1937, Freund developed an emulsion of water and mineral oil containing killed mycobacteria called Complete Freund's Adjuvant (CFA), which is a standout amongst the most intense adjuvants developed up to date (Freund et al., 1937). Despite being the gold standard adjuvant, CFA was shown to cause serious local responses and has since been classified as unreasonably dangerous for use in human and animal vaccines. Similar oil-in-water emulsion with no mycobacteria is known as Freund's Incomplete Adjuvant; it is less harmful and has been utilized in human vaccines formulations. During the 1950s, Johnson et al. confirmed that lipopolysaccharides (LPS) from Gram-negative bacteria displayed adjuvant activity (Johnson et al., 1956) but it is not used in human vaccines because it can trigger septicemia (Stefanova et al., 1993)

1.3.3 Synthetic adjuvants and second-generation delivery depot systems

A broad measure of work was finished during the 1990s undertaken to improve the adjuvant impacts of liposomes, PEG [poly(ethylene glycol)]lated lipids. PEG-modified lipids are typically added to phospholipid vesicles used for drug delivery to provide a steric coating on the surface of the membrane to hinder clearing of the particles by the reticuloendothelial system (RES) thereby prolonging the circulating plasma half-life. Utilization of liposomes for delivery of DNA vaccines has been considered. However, it is generally considered that liposomal delivery of subunit protein peptide antigen alone does not improvement of immunogenicity (Ott & Van Nest, 2007).

A second lipid-based particle with extensive application is the ISCOM/ISCOMATRIX system, based on Quillaja saponin, cholesterol, and phospholipids. Immunostimulatory complexes

(ISCOMs) are particulate antigen delivery systems composed of antigen, cholesterol, phospholipid and saponin, while ISCOMATRIX is a particulate adjuvant comprising cholesterol, phospholipid and saponin but without antigen. The combination of an antigen with ISCOMATRIX is called an ISCOMATRIX vaccine. ISCOMs and ISCOMATRIX combine the advantages of a particulate carrier system with the presence of an in-built adjuvant (Quil A) and consequently have been found to be more immunogenic, while removing its haemolytic activity of the saponin, producing less toxicity. Research has shown that Immune stimulating complexes (ISCOMs) are lipid-based particles that have shown potential as adjuvants and carriers for antigens. They have been used as prophylactic or therapeutic vaccines injected into the muscle as well as via mucosal and cutaneous administration. Both cellular and humoral immune responses have been reported after vaccination with antigens co-administered with ISCOM adjuvants (Nielsen et al., 2015; Ott & Van Nest, 2007).

1.3.4 Development of receptor-associated adjuvants that activate the innate immune system

Components of bacteria and viruses that are PAMPs that bind to PRRs on innate immune cells can act as vaccine adjuvants. For example, immunostimulatory CpG oligodeoxynucleotides (ODNs) are unmethylated cytosine-guanine dinucleotides found in bacterial DNA but absent in mammalian DNA (Aguilar & Rodriguez, 2007; Krieg, 2002). Overall, several hundred natural and synthetic compounds have been identified to have adjuvant activity (Krieg et al., 2001). CpG ODN bind to TLR9 causing simultaneous maturation of immature DC and activation of mature DC to produce cytokines and acts as adjuvants for Th1-type T cell responses and cytotoxic T cell responses (Sparwasser et al., 1998)

Although LPS is a very robust adjuvant, it is not considered safe for use in human vaccines because it can cause septicemia. Detoxified LPS or related compounds such as lipid A are a much

safer adjuvant alternative to LPS. Monophosphoryl lipid A has been used since 2009 in one vaccine in the US, Cervarix_(Einstein et al., 2009) and it has been tested for safety in tens of thousands of people and found to be safe. *Polyinosinic:polycytidylic acid* (Poly I:C) that stimulates TLR3. RIG1 and MDA-5 is another adjuvant that enhances immunity to vaccines and experimental antigens by a variety of mechanisms. The focal element of potency associated with Poly I:C is by binding to TLR3 hence directly activating DCs and type I IFNs through MDA-5 (Tritto et al., 2009)

1.4 Classification of adjuvants

Criteria for classifying adjuvants can include those that act as delivery systems and those that act as immunostimulatory adjuvants (Cox & Coulter, 1992). Adjuvants that act as delivery vehicles generally act as a depot and/or promote the slow release of antigen from the site of injection. This slow release leads to sustained stimulation of the immune system for production of elevated antibody titers. Many adjuvants such as alum, liposomes, emulsions, ISCOMs and numerous particulate vaccine adjuvants are thought to exert part of their adjuvant activity through depot formation (Cox & Coulter, 1997; Mosca et al., 2008)

Immuno-stimulatory adjuvants activate innate immune cells leading to secretion of cytokines and chemokines that enhance immune cell recruitment at the injection site, APC maturation and antigen presentation to effector T cells (Cox & Coulter, 1997). These adjuvants include CpG ODNs, muramyl dipeptide (MDP) and monophosphoryl A (MPL). Adjuvants such as the aluminum compounds (alum) have been dominantly used in many vaccines due to their immunopotential and safety records since 1920s. However, how these mineral agents influence the immune response to vaccination remains elusive. Many hypotheses exist as to the

mode of action of these adjuvants, such as depot formation, antigen (Ag) targeting, and the induction of inflammation. Recent advances in immunobiology have provided evidence that adjuvants initially characterized as delivery systems can actually activate innate immunity (Guy, 2007; Magiri et al., 2018b).

1.5 Adjuvants selection consideration

Some considerations for adjuvant choice includes the type of antigen type to be used, the species to be immunized, the injection route and the probability of adverse reactions (Byars & Allison, 1990; Gupta, 1998). Ideally, adjuvants should be steady with long shelf half-life, biodegradable, not induce immune responses against themselves and induce desired immune responses (for example humoral or cellular mediated depending upon prerequisites for protection) (Edelman, 1980) .

Reports demonstrate that aluminium is a weak adjuvant for induction of antibody responses to recombinant vaccines and prompts a Th2-type T cells, as opposed to a Th1-type T cells response (HogenEsch, 2012). Some other adjuvants including oil emulsions, lipopolysaccharides, polymers, saponins, liposomes, cytokines, ISCOMs, CFA, IFA, alums, bacterial poisons and so forth, have been assessed and clinical preliminaries are under scrutiny, in spite of the fact that MOA of adjuvants frequently remain inadequately studied (Edelman & Tacket, 1990). Safety is perhaps the single most important impediment in introducing most such adjuvants to human use. Synthetic adjuvants have numerous drawbacks, for example, side effects, severe injection site reactions and carcinogenesis, in addition to complicated preparations or inability to expand immunogenicity of the feeble antigen (Edelman & Tacket, 1990).

1.6 Adjuvants approved for human use

Not many vaccine adjuvants have been authorized for prophylactic use in human. Among them,

alum (aluminum salts) has been generally utilized for over 70 years and up to this point it is the main adjuvant approved in the United States. Oil-in water-emulsions (MF59 and AS03) are authorized for flu vaccines in Europe. AS04, a blend adjuvant made out of MPL adsorbed to alum is endorsed for HBV and HPV vaccines in Europe and has been as of late authorized in the USA (Mbow et al., 2010; O'Hagan & De Gregorio, 2009)

Table 1.2 Licensed adjuvants for human use

Adjuvant	Year licenced	Country/region	Company	Class	Indication
Alum	1924	United states	Various	Mineral salts (potassium aluminum sulfate, ammonium aluminum sulfate, and sodium aluminum sulfate. Potassium aluminum sulphate)	Various
MF59	1997	Europe	Novartis	Oil in water emulsion	Influenza (Fluad)/pandemic flu
ASO3	2009	Europe	GSK	Oil in water emulsion + α -tocopherol	Pandemic Flu (Pandemrix)
AS04	2000	Europe and United states	GSK	MPL + alum	HBV (Fendrix), HPV (Cervarix)
Liposomes	2005	Europe	Crucell	Oil in water emulsion	HAV, Flu (EU)

1.7 Mechanisms of Action

The MOA by which adjuvants mediate their responses may include depot formation at the site of injection leading to sustained release of antigen and continuous stimulation of immune system.

Water-in-oil emulsion adjuvants such as CFA create such depots (Herbert, 1968; Lambrecht et al., 2009). Other MOA include cytokines and chemokines upregulation, induction of recruitment of immune cells at the injection site, increased uptake and presentation of antigen to APCs and induced APC activation and maturation by increasing their expression of MHC class II and co-

stimulatory molecules (Cox & Coulter, 1997; Fraser et al., 2007; Hoebe et al., 2004).

Upregulation of cytokines and chemokines promote local immune cell recruitment at the site of injection causing uptake of the vaccine and antigen by the recruited immune cells that then traffic to the draining lymph nodes to induce adaptive immune responses. Alum, CpG, MF59 and PCEP have been shown to activate “adjuvant core response genes” which are genes expressed commonly for all three of these adjuvants (Awate, 2012; Magiri et al., 2016; Mosca et al., 2008).

Activation of adjuvant core response genes is known to increase cytokine-cytokine receptor interaction, host-pathogen interaction and defence immune protein activity (Mosca et al., 2008).

MF59 promotes recruitment of neutrophils, monocytes, macrophages and DCs to the site of injection in mice (Calabro et al., 2011). The adaptive immune response relies on antigen presentation on APCs. Although it is not fully understood how it works, antigen absorbs onto alum and interacts with lipids on DCs through an abortive phagocytosis then the DCs take up the antigen leading to antigen processing, presentation and DC activation (Calabro et al., 2011).

Although alum is able to induce antigen presentation by enhancing surface expression of MHC class II and costimulatory molecules on the surfaces of macrophages and monocytes, it does not directly affect APC maturation (Seubert et al., 2008; Sun et al., 2003). AS04 is able to stimulate DC activation through TLR4 pathway signalling (Didierlaurent et al., 2009). CpG ODNs can promote signaling in APCs through TLR9, LPS promotes signaling through TLR4 while Poly I:C activates TLR3 signaling (Chow et al., 1999; Klaschik et al., 2009; Klinman et al., 1996) which induces maturation and activation of DCs such as induced expression of costimulatory molecules and cluster of differentiation molecules. Cytokines such as IL-2, IL-6, IL-7, IL-13, IL-15 and hepatocyte growth factor (HGF), in combination or even, in some cases, alone, can contribute to the maturation of DCs (Didierlaurent et al., 2009; Kerkmann et al., 2003; Werninghaus et al., 2009).

The inflammasome is an important component of the innate immune response that can be a target for many adjuvants (Awate et al., 2013; Martinon et al., 2009). The inflammasome is expressed in myeloid cells and it is a complex assembly of proteins that are activated by NLRs family members (Awate et al., 2013) or DAMPs by components released from dying cells (Matzinger, 1994). Danger signals such as uric acid, nucleotides, adenosine triphosphate (ATP), reactive oxygen intermediates, and cytokines released from damaged cells can activate the inflammasome leading to release of pro-inflammatory cytokine such as IL-1 β (Kool et al., 2008). Molecules such as (Krysko et al., 2011). This sterile/semi-sterile inflammation can be induced in response to injection of adjuvants and release of these endogenous molecules can attract inflammatory cells and promote induction of innate immunity (Chen et al., 2007; Kono et al., 2014; Shi et al., 2003)

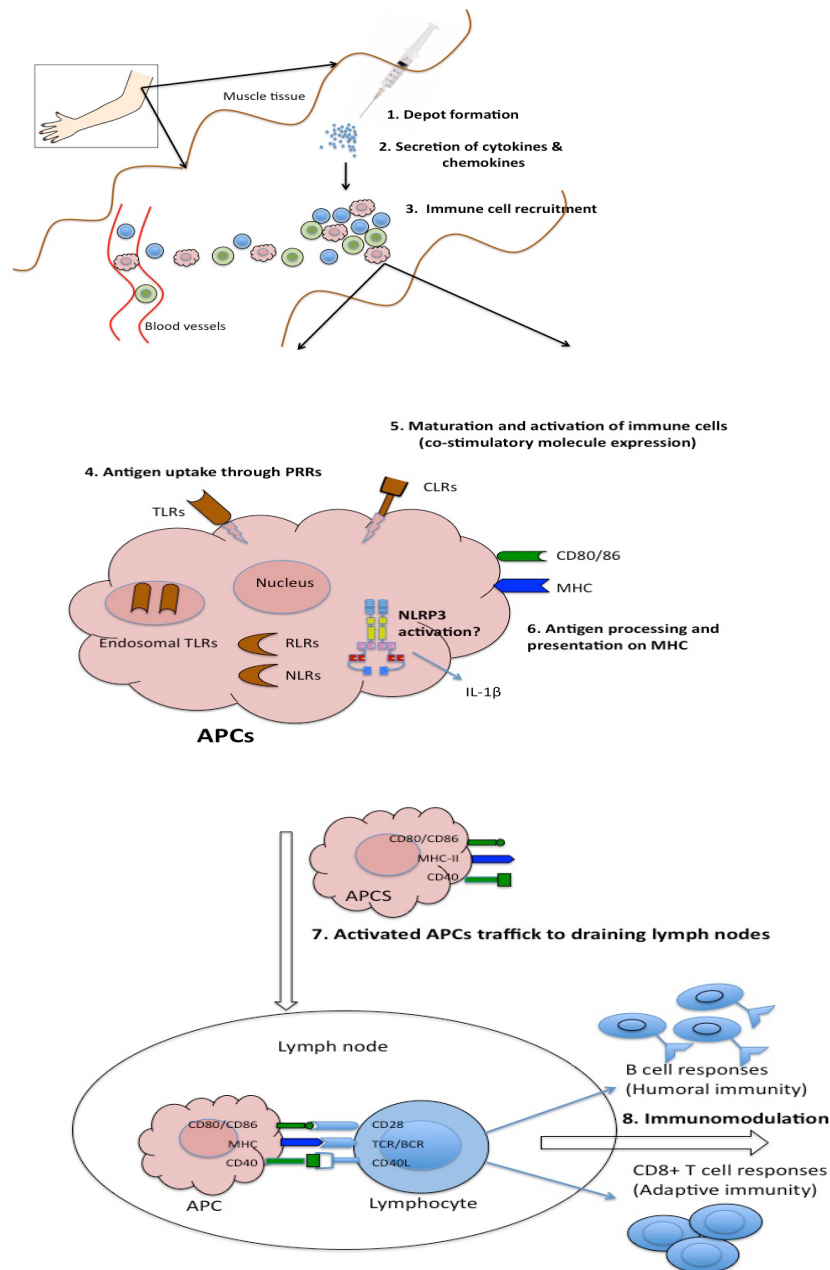


Figure 1.2 Diagrammatic representation of MOA of adjuvants: Adjuvants have a variety of mechanisms of action including forming a depot, secretion of chemokines and cytokines, and immune cell recruitment at the injection site, activation and maturation of antigen-presenting cells including activation of the inflammasome as well as shaping the type of immunity during antigen presentation in the lymph node (Awate et al., 2013).

1.8 How adjuvants link innate and adaptive immune responses

Janeway (1989) described adjuvants as “immunologist’s dirty little secret” (Janeway, 1989) which means that in spite of the wide utilization of adjuvants in billions of dosages of both human and animal vaccines, the MOA by which they potentiate their activity was not known at the time.

Recent advances in immunobiology have contributed to a greater understanding of the innate and adaptive type of immune responses. The innate immune system is acted upon by adjuvants binding to a number of conserved PRRs, such as the TLRs (Underhill & Ozinsky, 2002), NLRs, RLRs and CLR (Stahl & Ezekowitz, 1998). These receptors function by specific recognition of PAMPs and/or DAMPs (Medzhitov & Janeway, 1997) leading to activation of a variety of transcription and translation factors and a downstream immunological pathways which enhance genes and secretion of proinflammatory cytokines (Medzhitov & Janeway, 1997). Hence adjuvants that stimulate PRRs may enhance the protective immune response and promote memory to a specific antigen (Cox & Coulter, 1997)

Effective vaccines formulations need to stimulate multiple PRRs to both enhance the magnitude and the quality of immune responses to the vaccine antigens. Many adjuvants signal through PRRs on various immune cells resulting in increased NF- κ B. Proinflammatory cytokines and/or type I interferon (IFN) production, which subsequently leads to an up-regulation of chemokines and cytokines leading to recruitment of immune cells such as DCs and other APCs to the site of injection. The recruited DCs express various PRRs both on the surface (TLRs, CLR) and intracellularly (NLRs and RLRs), which are recognized and/or are activated by the adjuvants needed for maturation of dendritic cells (DCs) and improved antigen uptake and presentation to antigen presenting cells (APC), activation and maturation of APC [increased major histocompatibility complex (MHC) class II and co-stimulatory molecules expression]. Mature

APCs up-regulate the expression of MHC and co-stimulatory molecules. They are also characterized by increased capacity for antigen processing and presentation. Mature APCs then migrate to the draining lymph nodes to interact with antigen-specific B or T cell to activate potent antibody secreting B cells and/or effector CD8⁺ T cell responses (Cox & Coulter, 1997, Hoebe et al., 2004)

Therefore effective adjuvants can improve the immunogenicity of inactivated or recombinant antigens, and they can lessen the quantity of antigen per dose or the number of inoculations required to accomplish protective immunity. They may specifically improve the efficacy of vaccines in newborns, the elderly or immunocompromised persons, enhance the speed and duration of the immune response, modulate antibody avidity, specificity, isotype or subclass distribution, stimulate cell mediated immunity, promote the induction of mucosal immunity, and to help overcome antigen competition in combination vaccines (Rajput et al., 2007; Singh & O'Hagan, 2003).

1.9 Recent advances in experimental polyphosphazenes (PZs) adjuvants and their mechanisms of action (Cell and Tissue Research, December, 2018: 374(3), 465-471)

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1.9.1 PZs as immunostimulatory adjuvants

PZs are high molecular weight, water-soluble, synthetic polymers that have been shown to enhance the magnitude, quality and duration of immune responses when co-administered with

bacterial and viral antigens in mice, pigs and cattle (Andrianov et al., 2009; Andrianov et al., 2011; Andrianov et al., 2006; Dar et al., 2012; Eng et al., 2010; Garlapati et al., 2010; Garlapati et al., 2011; Magiri et al., 2018a; McNeal et al., 1999; Mutwiri et al., 2008; Mutwiri et al., 2007).

. The two most investigated PZs are poly [di(carboxylatophenoxy)phosphazene] (PCPP) and PCEP (Mutwiri & Babiuk, 2009a).

Changes in synthesis (such as reduction in the reduction of acid groups) and formulation as a soluble adjuvant or microparticle impacts how they influence the immune response (Andrianov et al., 2004). PCEP has been shown to have a significantly higher adjuvant activity compared to PCPP (Mutwiri et al., 2008) and also to induce 1000-fold higher antibody titres compared to alum when co-administered subcutaneously with an influenza antigen in mice (Mutwiri et al., 2007). Relative to PCPP, PCEP also promotes a significantly stronger mixed Th1/Th2 type T cell response leading to broad-spectrum immunity (Mutwiri et al., 2007).

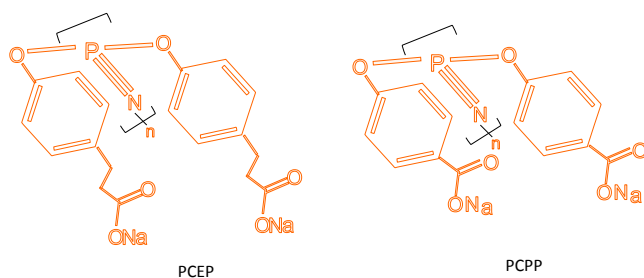


Figure 1.3 The structures of the polyphosphazenes adjuvants, PCEP and PCPP (Andrianov et al., 2006; Teasdale & Brüggemann, 2013). Image recreated from Motifolio.com

1.9.2 Regulation of innate immune response genes, induction of cytokines and chemokines, and recruitment of immune cells to the site of injection by PZs in mice and pigs

Studies with mice and pigs revealed species-specific differences in PZ-induced stimulation of innate immune responses (Awate et al., 2012; Magiri et al., 2016). Intramuscular injection of PCEP induced time-dependent changes in the gene expression of many “adjuvant core response genes” (Mosca et al., 2008) such as chemokine genes CCL-2, CCL-4, CCL-5, CCL-12 and CXCL-10 in mice (Awate et al., 2012) and CCL2 and CXCL10 (but not CCL- 5) in pigs (Magiri et al., 2016). Major transcription factor NF- κ B gene and the inflammatory cytokine TNF- α gene were up-regulated in response to PCEP in mice (Awate et al., 2012) but not in pigs (Magiri et al., 2016). At the protein level, PCEP promoted significant local production of Th1-type proinflammatory cytokines (IL- 1 β , IL-6, IL-18 IFN- γ and TNF- α) and Th2-type cytokines (IL-4 and monocyte chemoattractants CCL-2 and CXCL-10) at the site of injection in mice but not systemically (Awate et al., 2012). Further, *in vitro* studies showed that PCEP activated the NLRP3 inflammasome in a Caspase 1-depended manner which lead to the processing of interleukin IL-1 β , IL-18 and IL-33 stimulated splenic DCs in mice (Awate et al., 2014). However, in pigs, PCEP induced IL-6 gene expression but not IL-10, IL-17 or IFN- α (Magiri et al., 2016). PCEP injection in mice increased the expression of TLR4 and TLR9 at the site of injection (Awate et al., 2012) whereas PCEP did not induce significant expression any of the TLR genes in pigs suggesting differences in activation of immune responses in different animal species may be due to cell recruitment and differences in PPRs expression (Magiri et al., 2016). These results suggest that PCEP may modulate antigen-specific immune responses by activating early innate immune responses and promoting a strong immunostimulatory environment at the

site of injection. Our studies provide evidence that the effect that adjuvants have on the innate immune response can differ remarkably between species.

Intramuscular (i.m.) injection of PCEP promoted recruitment of largely neutrophils but also macrophages, CD4⁺ T cells, CD8⁺ T cells and CD19⁺ B cells, monocytes and DCs to the injection site and the draining lymph nodes in mice (Awate et al., 2014a). Confocal analysis revealed that many recruited myeloid cells (but only a few lymphocytes) showed evidence of intracytoplasmic lysosomal localization of PCEP (Awate et al., 2014b). These findings suggest that the recruitment of distinct immune cells to the site of injection site may be an important mechanism by which PCEP potentiates immune responses.

1.9.3 Activation of immune cells by PZs

Even in the absence of antigens, PCPP and PCEP have strong avidity to soluble immune receptor proteins such as Mannose Receptor and endolysosome membrane-associated PRRs such as TLR7, TLR8, and TLR9 (Andrianov et al., 2016a; Sasai & Yamamoto, 2013). Other studies revealed direct activation of immune cells by PCPP and PCEP through TLR signaling pathway, both on the external cell surface (TLR4) and endosome (TLR3 and TLR9) receptors (Reed et al., 2013; Sasai & Yamamoto, 2013). Incubation of primary mouse splenocytes with PCEP or PCPP triggered production of IL-4 and IL-12, but only PCEP induced significant IFN- γ production suggesting that activation of innate immunity may be important in mediating PZ adjuvant activity (Mutwiri et al., 2008). Others have demonstrated that PCPP induced activation and maturation of DCs (Andrianov et al., 2006; Andrianov et al., 2016a). In the presence of antigen, PCPP has been shown to promote activation and maturation of human adult and newborn DCs by upregulating co-stimulatory molecules and cytokine production and was as induction of an innate immune

transcriptome (Palmer et al., 2014) which may suggest that PZ may be an appropriate adjuvant to include in early life immunization.

1.9.4. Vaccine carrier adjuvants

Vaccine carriers have been traditionally viewed as particulate delivery vehicles capable of facilitating physical uptake of the antigen by APCs (De Temmerman et al., 2011; Storni et al., 2005). Generally, it was thought that delivery systems tend to induce Th2-type immune responses which are not effective against many intracellular pathogens, while immunostimulatory adjuvants were traditionally thought to induce Th1-type immune responses by strongly activating the innate immune system (Ryan et al., 2001). However, these classifications are no longer appropriate since there is growing evidence that some delivery systems can activate innate immunity as well.

1.9.4.1 PZs as vaccine carriers

PZs have been exploited as protein carriers due to their versatile molecular structures and wide-spectrum of chemical and physical properties including biodegradability and matrix permeability (Andrianov & Payne, 1998; Teasdale & Brüggemann, 2013). PZ can bind vaccine antigens as well as TLR ligands or other sites on immune cells leading to cell maturation and more effective antigen processing which supports the idea that PZs macromolecules have dual antigen carrier and immunostimulant functions (Andrianov et al., 2016a; Andrianov et al., 2005; Palmer et al., 2014). Further, PZs can form stable water-soluble, complexes with antigenic molecules spontaneously and thus do not require chemical conjugation (Andrianov et al., 2005; Palmer et al., 2014). These non-covalent interactions with proteins stabilize proteins in solution and during drying processes and they have been correlated with immunoadjuvant activity (Andrianov et al., 2005; Marin et al., 2010).

Aqueous PZs can be transformed to microparticles by cross-linking them with divalent cations. Microencapsulation of antigens by PZs can be achieved under remarkably mild physiological conditions (which avoids denaturation or loss of biological activity of encapsulated material) giving them tremendous potential as matrices for sustained antigen release (Andrianov & Payne, 1998). For example, immunogenicity of influenza antigen and tetanus toxoid were dramatically enhanced when microencapsulated in PCPP microparticles (Payne et al., 1995). Further, by varying polymer ratios and using PZ of reduced molecular weight, it can form macromolecular assemblies at the nanoscale level to cross-linked hydrogels while maintaining protein-binding ability (Andrianov et al., 2016b). Microparticles are more effective in mucosal delivery of antigens (Shim et al., 2010) which should be taken into consideration for vaccine development.

1.9.5 Adjuvant potential of PZs in combination with other adjuvants

Due to challenges in vaccine development, increased regulatory hurdles and/ for purely economic reasons, the vaccine industry has historically used one adjuvant per vaccine. However, evidence has accumulated over the last decades that multiple adjuvant components in the same vaccine may act synergistically (Ciabattini et al., 2016; Didierlaurent et al., 2017; Kindrachuk et al., 2009; Levast et al., 2014; Madan-Lala et al., 2017; Mount et al., 2013; Mutwiri et al., 2011; Salvador et al., 2012). Combination adjuvants are particularly suited to only enhance and/or direct the immune responses towards a Th1-, Th2- or Th17-type immune responses (Kindrachuk et al., 2009; Levast et al., 2014; Salvador et al., 2012).

Due to the short half-life of most immunostimulatory adjuvants *in vivo*, combining a delivery vehicle adjuvant with an immunostimulatory adjuvant may increase the magnitude and modulate the quality of immune responses (Weiner et al., 1997). Mice vaccinated subcutaneously with

PCPP microparticles encapsulating OVA and CpG ODN generated higher antigen-specific antibody responses compared to antigen alone (Garlapati et al., 2010; Wilson et al., 2010). Studies by several investigators at VIDO-InterVac have demonstrated that PZ as part of a triple adjuvant combination (TriAdj) consisting of PCEP or PCPP plus TLR agonist (CpG or polyIC) plus Host Defense Peptide (HDP) is a robust adjuvant combination in multiple species and multiple routes of delivery. For example, subcutaneous immunization of mice with of hepatitis B surface antigen (HBsAg) plus TriAdj resulted in enhanced production of HBsAg-specific antibody responses compared with the mice immunized with HBsAg plus any of the three adjuvants alone (Mutwiri et al., 2008). Relative to mice immunized with OVA plus the adjuvants alone, mice vaccinated with OVA plus TriAdj showed enhanced antibody and cell mediated responses via both MHC class I and II pathways, promoting a more balanced antibody-mediated and type1-biased cell-mediated immune response (Kovacs-Nolan et al., 2009a). Mice vaccinated subcutaneously with *Bordetella pertussis* antigen plus TriAdj had significantly reduced bacterial load after challenge and increased antigen-specific IL-17 secreting cells relative to vaccine comprised of one or two adjuvants alone (Garlapati et al., 2011). Formulation of pertussis toxoid (PTd) with TriAdj increased IgG1 responses in adult mice and induced superior serum IgG2a antibody titers in both adult and neonatal mice compared to mice immunized with each adjuvants alone (Gracia et al., 2011). Recombinant truncated bovine respiratory syncytial virus (BRSV) fusion protein (DeltaF) plus TriAdj showed enhanced secretion of antigen-specific serum antibody titres when compared with mice immunized with antigen alone (Kovacs-Nolan et al., 2009b). Intranasal vaccination with a formalin-inactivated BRSVs vaccine plus TriAdj resulted induced systemic and mucosal immunity in mice (Mapletoft et al., 2010) and significant reduction in viral replication upon BRSV virus challenge (Mapletoft et al., 2008). Cattle immunized subcutaneously on days 0 and 90 with TriAdj with hen egg lysozyme antigen

produced superior antigen-specific humoral responses and cell-mediated immune responses relative to cattle immunized with Emulsigen (Kovacs-Nolan et al., 2009c). Intramuscular or intrauterine immunization of rabbits with a single dose of OVA, truncated glycoprotein D (tgD) from bovine herpesvirus, and a fusion protein of porcine parvovirus protein VP2 and bacterial thioredoxin (rVP2-TrX) formulated with TriAdj induced antigen-specific humoral responses systemically and within the local (uterus) and distal mucosa (lungs and vagina) (Pasternak et al., 2017). Thus, PZ as part of the TriAdj combination dramatically enhanced the magnitude of immune responses resulting in a balanced immunity for broader protection.

1.9.6 Antigen dose sparing effect of PZs adjuvants

The implementation of antigen stabilization and dose-sparing technologies is an important step in improving availability of vaccines and is a critical feature for effective vaccine development at the time of a pandemic outbreak. PZ have the potential to significantly reduce the cost of vaccination by reducing the number of immunizations or reducing the minimal doses of antigen required to induce significant immunity. Indeed, lethal challenge studies in ferrets demonstrated 100% protection for low-antigen dose PCPP-adjuvanted formulations and at least a 10-fold antigen-sparing effect with improved thermal stability of H5N1 influenza vaccine in solutions (Andrianov et al., 2011). Additionally, reducing the dose of antigen by 25-fold had no effect on antibody responses in mice immunized with PCPP and PCEP in mice (Mutwiri et al., 2007). When used as part of an intradermal delivery system for hepatitis B surface antigen, PCPP demonstrated superior induction of immunity in pigs compared to i.m. administration and significant antigen sparing potential (Andrianov et al., 2009). Further development of PZ as an adjuvant may therefore have a great impact in the vaccine industry.

1.9.7 The safety profile of PZs adjuvants

Many potential immunological adjuvants are not licensed for use in humans or veterinary species due to safety and/or toxicity concerns (Eng et al., 2010; Petrovsky, 2015; Sivakumar et al., 2011). PZs have been shown to be a safe and effective adjuvant at doses up to 1 mg when injected in sheep and cattle (Kovacs-Nolan et al., 2009c) without adverse reactions such as pathological inflammatory reactions, swelling or pain, (Kovacs-Nolan et al., 2009c). In pigs, up to 500 µg PCEP was tolerated with fewer injection site reactions and reduced delayed type hypersensitivity in pigs (Dar et al., 2012; Magiri et al., 2016; Magiri et al., 2018a).

In human Phase I clinical trials for three influenza viral strains (A/H3N2, A/H1N1 and B strain) targeted towards both young and elderly adults, up to 500 µg PCPP was shown to be safe showing sterile abscesses, and non-ulcerative necrosis at the site of inoculation. (Le Cam et al., 1998). Phase I and Phase II clinical trials of a vaccine formulated with PCPP and HIV-1 antigens did not result in either abscess at injection site, immune dysfunction, anaphylaxis, or allergy, whereas a vaccine formulated with CFA and HIV-1 was associated with definable long-term adverse events (Gilbert et al., 2003). Together, the results suggest that polyphosphazenes are well tolerated in humans and animals but detailed safety and toxicity studies per vaccine are still required.

CHAPTER 2. RATIONALE, HYPOTHESIS, OVERALL OBJECTIVE AND AIMS

2.1 Rationale and hypothesis

Vaccination continues to be a very important public health tool in the control of infectious diseases in the world. Subunit vaccines containing even inactivated antigens consequently are ineffectively immunogenic and require the addition of adjuvants to generate protective immune responses. Despite their crucial role, the MOA of numerous adjuvants remain ineffectively understood. The lack of detailed information on how adjuvants work is a barrier to their rational use in vaccines especially the development of safe and effective vaccines. Although adjuvants have been used in billions of doses of vaccines, little is known regarding some of their MOA.

PZs are high-molecular weight, water-soluble polymer that have been shown to enhance long lasting immune responses with a variety of viral and bacterial antigens in mice pig and cattle (Mutwiri et al., 2008, McNeal et al., 1999, Eng et al., 2010). The two most investigated PZs are PCPP and PCEP; PCEP has been shown to possess significantly stronger adjuvant activity than PCPP because of its ability to promote mixed Th1/Th2 type of immune responses hence giving a broad spectrum immunity (Mutwiri et al., 2007). Recent advances in immunobiology in mice suggest that most adjuvants including PCEP act by stimulating the innate immune response leading to induction of cytokine and chemokine production at the site of injection (Mutwiri et al., 2008), recruitment of immune cells and transport of antigen to the draining lymph node (Awate et al., 2014a), increased antigen uptake by DCs and activation and maturation of antigen presentation cells. It was recently reported that some adjuvants injected into murine muscle induced a set of at least 56 "adjuvant core response genes" at the site of injection (Awate et al., 2012). *In vitro*, PCEP induces secretion of innate cytokines IL-12 and IFN- γ suggesting that activation of innate immunity may be crucial in mediating its adjuvant activity (Mutwiri et al.,

2008). These studies were carried out in mice and there is little information as to its MOA in large animals.

Since most adjuvants act by stimulating innate immunity, we will focus on investigating how PCEP activates innate immunity as a means to understand its mechanism of action in pigs.

Hypothesis

1. PCEP is a strong activator of innate immune responses (inflammation) in pigs.
2. Strong inflammatory responses are not required for adjuvant activity of PCEP.

2.2 Overall Objective

The overall objective was to investigate innate immune responses activated by adjuvant PCEP in pigs by addressing the following specific aims

2.3 Aims

1. To investigate the immune response genes induced by PCEP at the injection site
2. To determine inflammatory responses induced by PCEP at the injection site and in the draining lymph node
3. To investigate the local and systemic cytokine and chemokine concentrations after PCEP injection
4. To examine whether reduction of dose of PCEP reduces inflammatory responses at the site of injection
5. To determine whether reduction in dose of PCEP reduces tissue reaction to the vaccine without compromising the immune response to swine influenza virus antigen.

CHAPTER 3. Response of immune response genes to adjuvants poly [di (sodium carboxylatoethylphenoxy) phosphazene](PCEP), CpG Oligodeoxynucleotide and Emulsigen at intradermal injection site in pigs

(Veterinary Immunology Immunopathology 2016. 175: 57-63)

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Relationship of this study to the dissertation

Adjuvants are critical component of vaccines. Despite being a critical component of many vaccines, the MOA are not fully understood and particularly in regard to large animals. This lack of understanding limits our ability to design effective vaccines. PCEP has shown great potential as a candidate for an experimental vaccine adjuvant with different viral and bacterial antigens in mice pigs and cattle. However, the MOA of PCEP is not well studied. In this study, we investigated the capacity of a novel adjuvant PCEP to induce “adjuvant core response genes” (cytokines, chemokines, innate immune receptors, interferon-induced genes and adhesion molecules) when injected intradermal in pigs. This study suggests that PCEP enhance its adjuvant activity by strongly activating early innate immune responses genes that may induce immune cell recruitment which promote a strong immuno-stimulatory environment at the site of injection.

In this manuscript, my primary role was conduct of data analysis and interpretation of the work which led to the paper, writing the initial draft of the manuscript, and revising it based on

feedback from the other authors. Additionally, I responded to reviewer's comments appropriately before final publication.

3.1 INTRODUCTION

Vaccination continues to be a very important public health tool to control infectious diseases in human and animals (Drummond et al., 2007). Many modern vaccines contain highly purified antigens, which tend to be poorly immunogenic and therefore require the addition of adjuvants to induce protective immune responses. Recent evidence suggests that adjuvants may mediate their effects by modulating a variety of innate immune events including the production of cytokines and chemokines, the recruitment of immune cells, the enhancement of antigen uptake and presentation, and the promotion of antigen transport to draining lymph nodes. The type of innate response induced by adjuvants can impact the quality and quantity of adaptive immune responses. Understanding the mechanisms by which adjuvants mediate their effects will provide critical information on how innate immunity influences the development of adaptive immunity and may lead to the rational development of new, safe, and effective vaccines in large animal species.

PZs adjuvants are high-molecular weight, water-soluble polymers that, when coupled with many viral and bacterial antigens, have been shown to enhance long-lasting immune responses (Eng et al., 2010; Garlapati et al., 2010; McNeal et al., 1999; Mutwiri et al., 2008). When co-administered with influenza virus X:31 antigens, PCEP has been shown to induce a robust Th1/Th2 type, broad spectrum immune response in mice (Mutwiri et al., 2007). Other studies have demonstrated that PCEP promotes the induction of cytokine and chemokine

production (Awate et al., 2012; Mutwiri et al., 2008), which leads to immune cells being recruited to the injection site, as well as the transport of antigens to draining lymph nodes. Results from *in vitro* experiments with splenic DCs indicate that PCEP stimulated significant production of the innate immune response cytokines IL-12 and IFN- γ (Garlapati et al., 2011; Mutwiri et al., 2008). PCEP has been shown to activate B-cells and CD4⁺ and CD8⁺ T cells respectively, leading to a significant production of IgM and the induction of IFN- γ (Awate et al., 2014b). Our research group recently reported that CpG ODN and PCEP induced expression of a number of ‘adjuvant core response genes’ at the intramuscular injection site in mice (Awate et al., 2012). These data suggest that the powerful adjuvant activity of PZs and CpG are a consequence of the strong activation of innate immunity (Mutwiri et al., 2008).

To our knowledge, no studies have explored how a PCEP adjuvant mediates its activity in large animals. To obtain a better understanding on the adjuvant’s mechanism of action in large animals, we evaluated how the adjuvants PCEP, CpG ODN and Emulsigen injected intradermally into pigs modulated local expression patterns of immune response genes.

3.2 Materials and Methods

3.2.1 Animal experiments

Four groups (n=6/group) of three to four weeks old commercial cross breed pigs were administered a 250 μ l volume i.d. injection at the subcutis of phosphate-buffered saline (PBS), 100 μ g PCEP, 10 μ g CpG 2395, or 20% Emulsigen. Each animal was injected at five different injection sites five centimeters apart and an 8 mm skin punch plug biopsy was collected from one site at each time point (1, 6, 24, 48 and 96-hours). The animal experiments were approved by the University of Saskatchewan’s Animal Research Ethics Board for humane animal use.

3.2.2 Adjuvant preparation

PCEP was synthesized as detailed in (Awate et al., 2012). ssRNA CpG 2395 (Merial Pharmaceuticals, CA, USA) and Emulsigen (MVP Laboratories, OM, USA) were dissolved in endotoxin-free, sterile PBS at the pH 7.4 (Life Technologies, Carlsbad, CA, USA) prior to injection.

3.2.3 Quantitative real-time PCR (qRT-PCR) for gene expression

Skin tissue biopsies were collected at each time point for total RNA isolation using TRIzol (Life Technologies, Carlsbad, CA, USA) as per the manufacturer's instructions with the addition of a second isopropanol (Commercial Alcohols, Inc., Brampton, ON, Canada) precipitation to remove phenol and other contaminants. The biopsies were homogenized as detailed in (Awate et al., 2012).

RNA was quantified and checked for quality by using an Agilent RNA 6000 nano kit 5056-4476 (Agilent Technologies, Waldbronn, Germany) and run on the Agilent Bioanalyser. Before cDNA synthesis, the RNA samples were treated with DNase I (Life Technologies) for 20 minutes at room temperature. One milligram of the total RNA was used to synthesize cDNA.

The cDNA was prepared by using random hexamer and oligo(dT) primers and Superscript III reverse transcriptase (Life Technologies) using the manufacturer recommended concentrations. cDNA was then diluted in nuclease-free water to 10 ng/ μ L equivalent cDNA. qRT-PCR was performed in duplicate using 20 ng of equivalent cDNA, KappaFast Universal Mastermix (Kappa Biosystems, Wilmington, MA USA) with a primer concentration of 1 μ M. The differential expression of selected cytokine and chemokine genes was assessed using the Bio-Rad iCycler detection system (Hercules, California, USA). The specificity of the amplified

products was determined by visualising on a 1.8% Agarose gel (Life Technologies). The melt curve was used to test the primer product specificity by determining whether primer pairs (Table 1) were amplifying a single product (data not shown). As controls, we included a 'No Template Control (NTC)' reaction, wherein no cDNA was included, and a 'No RT control', where RNA samples were not reverse transcribed (data not shown). The PCR for the housekeeping genes β -ACTIN, RPL-19, and HPTR1 and the genes of interest from each sample were run in parallel over several PCR plates. The PCR efficiency for the primer probe set was evaluated against a serial dilution of pooled samples and found to be greater than 95% for all genes (data not shown). Target gene expression Ct values were first normalised using the iCycler iQ™ real-time PCR detection system as outlined in (Vandesompele et al., 2002). The increase in target gene expression levels in stimulated skin tissues were calculated as the fold change increase ($2^{-\Delta\Delta CT}$) relative to PBS and normalized against the geometric mean of the time-matched HPTR1 gene, which was the most stable for all time points.

Table 3.1 List of primers

Gene symbol	Forward sequence (5'-3')	Reverse sequence (5'-3')
CCL-2	AGTCACCTGCTGCTATACAC	GCGATGGTCTTGAAGATCAC
CCL-5	TGCCCTTGCTGTCATCCTC	CACACCTGGCGGTTCTTTC
CXCL-10	AGAACTGTTGCTGTACC	CATGTGGGCAAGATTGAC
IL-17	CTCTCGTGAAGGCGGGAATC	GTAATCTGAGGGCCGTCTGG
IFN- γ	GCTCTGGGAAACTGAATGAC	TCTCTGGCCTTGGAACATAG
IFN- α	CTCCTGGCACAAATGAGGAG	CTGAAGAGCTGGAAGGTCTG
IL-1 β	AGAAGAGCCCATCGTCCTTG	GAGAGCCTTCAGCTCATGTG
IL-6	ATCAGGAGACCTGCTTGATG	TGGTGGCTTTGTCTGGATTC
TNF- α	CCAATGGCAGAGTGGGTATG	TGAAGAGGACCTGGGAGTAG
IL-10	GGTTGCCAAGCCTTGTCAG	AGGCACTCTTCACCTCCTC
IL-13		
NF-kappa B	GGCCATCAATCAGCGCATCCAG	AGCAGAGCCGCACAGCATTCAG
TLR4	TGTGCGTGTGAACACCAGAC	AGGTGGCGTTCCTGAAACTC
TLR9	GGCCTTCAGCTTCACCTTGG	GGTCAGCGGCACAAACTGAG
IFIT2	CAGAAGGCGGCAGAGAATG	ACAGAGGCAGGCGAGATAG
IFIT3	CTCGCCAAACAGATGTCC	GGATTCCAGTGCCGTTAG
IFNAR2	TCCGCCTGGGTATTTCTTG	CGCTATCGCTGTCGCTTTC
MX1	AGTGTGCGCTGTTTACCAAG	TTCACAAACCCTGGCAACTC
MX2	CCGACTTCAGTTCAGGATGG	ACAGGAGACGGTCCGTTTAC
IRF7	TGCGATGGCTGGATGAAG	TAAAGATGCGCGAGTCGG
LGAL3	GACCCAAGGCGTGAAAGAC	CGTTCCTGAAGCCCAAAGC
PMSB8	TTGTGGCGGTGGATTCTC	AGCAGACGCTCCCAATAC
STAT2	CCATCTCTGGCACCTTTC	GAGCACCTTGTCGTCATC
MSR1	GATCAACCGGAGGACACTGAC	GCACCAGCACGACAAACAC
β -ACTIN	CCTCTCAGGCATGGAGTCCT	CTGATGTCCACGTCGCACTT
RPL19	AACTCCCGTCAGCAGATCC	AGTACCCTTCCGCTTACCG
HPRT1	GGACTTGAATCATGTTTGTG	CAGATGTTTCCAAACTCAAC

3.2.4 Statistical analysis

Statistical analyses were carried out using Graph-Pad Prism 6 software (GraphPad Software, San Diego, CA, USA). Differences in the fold change of gene expression were

identified using a non-parametric Kruskal-Wallis ANOVA test where Dunn's multiple comparisons test was used post-hoc to identify statistically significant differences in gene expression. The letter code depicting significant differences in gene expression between groups are as follows: (a) CpG to PBS, (b) Emulsigen to PBS, (c) PCEP to PBS, (d) CpG to Emulsigen, (e) Emulsigen to PCEP, and (f) CpG to PCEP. Differences were considered statistically significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) which are stated in the text.

3.3 Results and Discussion

3.3.1 Induction of chemokines, cytokine and Toll like receptor (TLR) genes expression in response to adjuvants

Awate *et al* (2012) reported that CpG and PCEP induced expression of a set of adjuvant core response genes when injected into murine muscle (Awate et al., 2012). Using a similar experimental design, we injected adjuvants into pigs via an intradermal route and included Emulsigen as another adjuvant under investigation. Please note that unless specifically stated, statistically significant data will always indicate the adjuvant relative to PBS at each time point.

3.3.1.1 Chemokines

Chemokine (C-C motif) ligand (CCL-2) is a chemokine with chemotactic activity for monocytes and basophils (Wegscheider et al., 2005). Other studies have shown that the adjuvants alum, MF59, and CpG-ODN modulated a cluster of genes encoding cytokines, chemokines, innate immune receptors, interferon-induced genes and gene encoding adhesion molecules defined as 'adjuvant core response genes' when administered to the injection site in mice

following intramuscular administration in quadriceps muscles (Mosca et al., 2008). Similarly, the oil-in-water emulsion AS03 co-administered with antigen to stimulate colony-stimulating factor 3 (CSF3) induced expression of chemokines CCL-2, CCL-3 and CCL-5 *in vitro* in mice dendritic cells (Morel et al., 2011).

In the present study, CCL2 was significantly induced in response to Emulsigen at 24 (b;***), 48 (***) and 96 hours (**) and it was significantly induced by PCEP at 96 hours (c; *) (Fig 4A). Thus, even though the magnitude was less robust in pigs our results show general agreement with results from Awate *et al* (2012) where PCEP was shown to induce CCL-2 expression >100 fold after 24 hours, rising to > 400 fold after 96 hours in murine muscle (Awate et al., 2012). CpG failed to significantly induce expression of CCL-2 in pig skin or murine muscle over time (Awate et al., 2012).

The chemokine CCL-5 activates T cells through a tyrosine kinase pathway (Bacon et al., 1995; Wong & Fish, 1998), which leads to the secretion of IFN- γ (Appay et al., 2000) and may promote dendritic cell maturation (Fischer et al., 2001; Yamagami et al., 2005). In the present study, CCL-5 expression was significantly induced by Emulsigen at 24 (b; **), 48 (*) and 96 hours (***; Fig 4B). Intradermal injection of CpG and PCEP into pigs failed to significantly increase expression of CCL-5 at any time point. In contrast, Awate *et al* (2012) showed that PCEP injection into murine muscle up-regulated the expression of CCL-5 gene at 24 hours whereas CpG injected into murine muscle was shown to promote CCL-5 expression at 12 and 48 hours (Awate et al., 2012).

CXCL10 binds and activates the CXCR3 receptor which is highly expressed in activated Th1 cells, B cells, NK cells, and DCs (Dufour et al., 2002). In the current study, CXCL-10 was significantly induced by CpG relative to both PBS and PCEP 24 hours (a; **, f; **) and 48 hours (a; ***, f; *) (Fig 4C). CpG induced a significantly higher expression of CXCL-10 relative to

Emulsigen at 6 hours (d; ***) but CXCL-10 expression rebounded to be induced in response to Emulsigen after 24 hours. Awate *et al* (2012) showed that murine muscle injected with CpG showed >500 fold induction of CXCL-10 after 6 and 48 hours and muscle injected with PCEP showed significant induction of CXCL-10 after 48 and 96 hours (Awate et al., 2012).

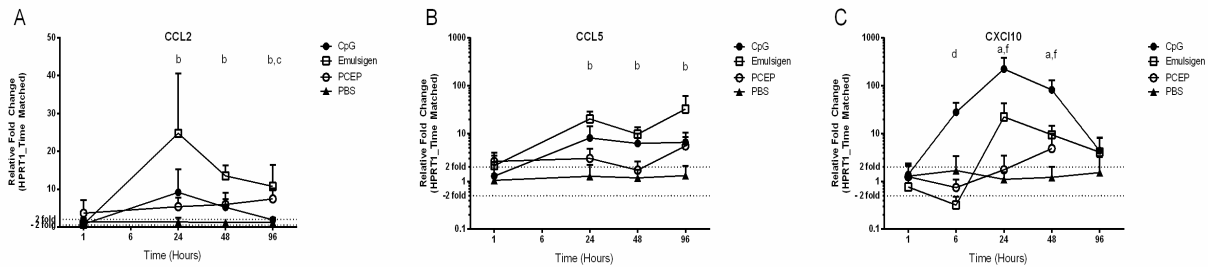


Figure 3.1 Chemokines gene expression profiles in response to PCEP, CpG and Emulsigen at the site of intradermal administration in pigs. Skin punch biopsies were collected at 1, 6, 24, 48 and 96 hours for chemokine genes expression by quantitative real-time PCR for CCL-2 (A), CCL-5 (B), and CXCL-10 (C). Results shown are the mean \pm SD of three to six replicates at each time point. Relative fold changes (y-axis) for each gene were calculated by the Ct method and are relative to the gene expression in PBS injected skin tissue and each gene was normalized to pig HPRT1 for each time point. The letter code depicting significant differences in gene expression between groups are as follows: (a) CpG to PBS, (b) Emulsigen to PBS, (c) PCEP to PBS, (d) CpG to Emulsigen, and (f) CpG to PCEP.

3.3.1.2 Cytokines

IL-6 is a proinflammatory cytokine that promotes the differentiation of B cells (Helle et al., 1988; Hilbert et al., 1989; Houssiau et al., 1988; Lue et al., 1991). In the current study, IL-6 was significantly induced by Emulsigen at 6, 24 and 48 hours (b; *, *** and *, respectively). PCEP induced IL-6 expression after 48 and 96 hours (c; ***, **, respectively) and relative to CpG after 96 hours (f; *) (Fig 5A). CpG did not promote IL-6 expression at the site of injection in pigs. These results are in agreement with Awate *et al* (2012), which showed that PCEP

injected in murine muscle was a more potent activator of cytokine genes IL-6 at the site of injection (which peaked at >400 fold at 24 hours) compared to CpG (Awate et al., 2012). Tumor necrosis factor- α (TNF- α) is a cytokine involved in acute-phase inflammation and is produced chiefly by activated macrophages as well CD4⁺ lymphocytes, NK cells, neutrophils, mast cells and eosinophils (Brynskov et al., 2002; Locksley, Killeen, & Lenardo, 2001). TNF- α was induced less than 10 fold by Emulsigen at 24, 48 and 96 hours (b; **, **, *, respectively) whereas CpG induced TNF α expression after 6 hours (a; *) (Fig 5B). Awate *et al* (2012) showed that injection of CpG into murine muscle triggered a transient increase in TNF α expression at 3-6 hours followed by reduced expression and finally increased expression again after 48 hours (Awate et al., 2012). Murine muscle injected with PCEP showed increased TNF α expression over time (Awate et al., 2012). There was no significant change in NFkB expression in pig skin in response to injection of any of the adjuvants (Fig 5C).

CpG injection into pig skin significantly induced expression of the potent anti-inflammatory cytokine, IL-10, after 24 and 48 hours (a; **, *) but no other adjuvant significantly induced expression of this gene in pigs (Fig 5D). IL-13 is an anti-inflammatory cytokine and a mediator of allergic inflammation (Cocks et al., 1993; Wynn, 2003). In the present study, IL-13 was significantly repressed in response CpG relative to Emulsigen at 24 hours (d; *) and relative to PCEP at 48 hours (f; **) (Fig 5E). In contrast, Awate *et al* (2012) showed that CpG and PCEP injected into murine muscle triggered increased IL-13 expression over time. Finally, our results in pigs showed that CpG significantly reduced expression of IL-17 after 24 hours (a; *) (Fig 5F). In contrast, Awate *et al* (2012) showed that in murine muscle, PCEP upregulated Th17 type cytokines (IL-17 and IL-6) and Th2 type cytokines (IL-4, IL-10, and IL-13) with expression generally increasing over time. Our data shows that in pig skin, adjuvants PCEP and promote Th2 stimulation through induction of IL-13 expression but that CpG inhibited IL-13 and IL-17

expression and it induced expression of IL-10. Together these data suggest that these adjuvants do not share conserved expression profiles of innate immune response genes in pig skin relative to murine muscle.

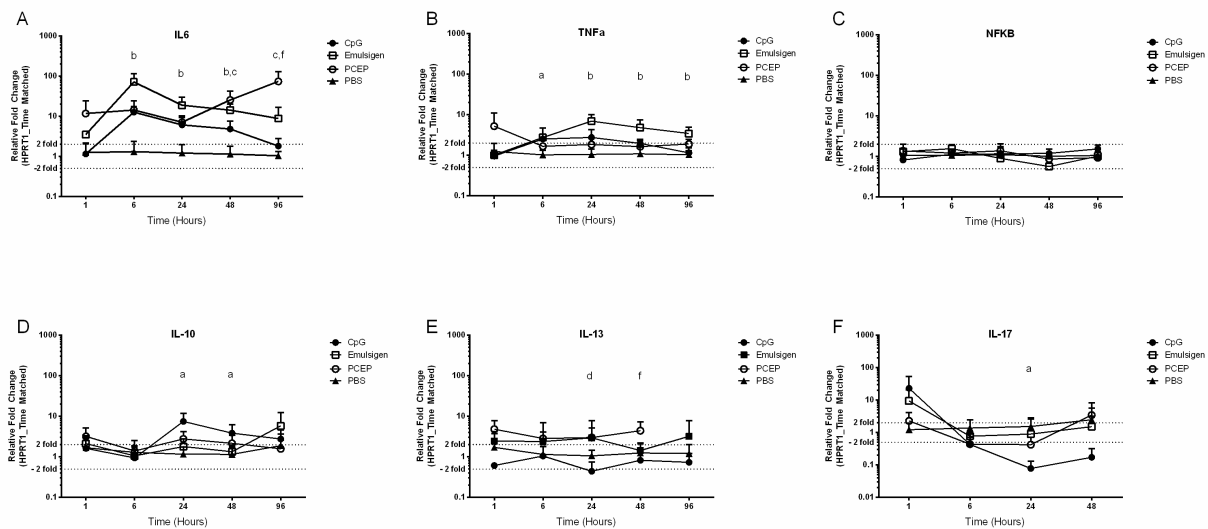


Figure 3.2 Induction of cytokine gene expression profiles in response to PCEP, CpG and Emulsigen at the site of intradermal administration in pigs. Skin punch biopsies were collected at 1, 6, 24, 48 and 96 hours and subjected to quantitative real-time PCR to quantify gene expression levels for IL-6 (A), TNF- α (B), NFKB (C), IL-10 (D), IL-13 (E) and IL-17 (F). Results shown are the mean \pm SD of six replicates at each time point. Relative fold changes (y-axis) for each gene were calculated by the Ct method and are relative to the gene expression in PBS injected skin tissue and each gene was normalized to pig HPRT1 for each time point. The letter code depicting significant differences in gene expression between groups are as follows: (a) CpG to PBS, Emulsigen to PBS, (c) PCEP to PBS, (d) CpG to Emulsigen, and (f) CpG to PCEP.

3.3.1.3 Toll-Like Receptors

The proteins coded for by the Toll-Like Receptors TLR4 and TLR9 genes are members of the TLR family (Du et al., 2000), which recognizes LPS and unmethylated CpG DNA/oligodeoxynucleotides, respectively and they trigger the activation of an innate immune response (Medzhitov & Janeway, 1997). In the present study, none of the adjuvants significantly induced expression of the TLR4 gene in response to PBS (Fig 6A) but the TLR9 gene was induced by Emulsigen after 24 hours (b; ***) and 48 hours (***). No other adjuvant induced expression of TLR9 induction at any other time point (Fig 6B). In contrast, Awate *et al* (2012) showed that murine muscle injected with PCEP up-regulated TLR4 and TLR9 at the injection site (Awate et al., 2012).

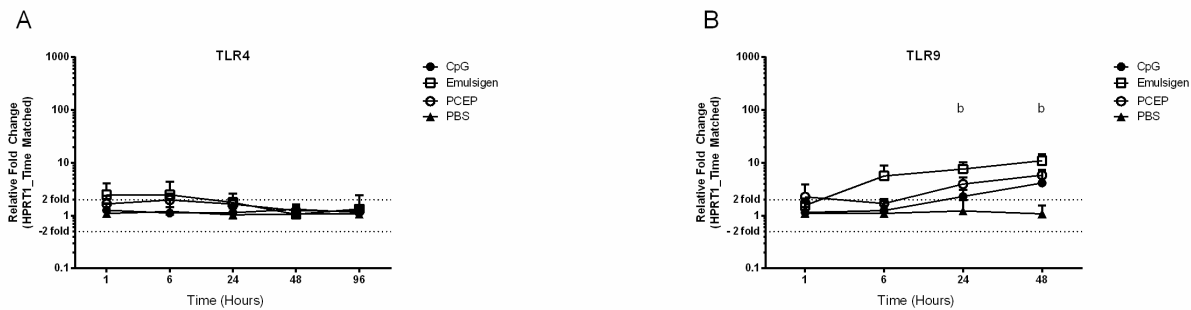


Figure 3.3 Induction of TLR gene expression profiles in response to PCEP, CpG and Emulsigen at the site of intradermal administration in pigs. Skin punch biopsies were collected at 1, 6, 24, 48 and 96 hours and subjected to quantitative real-time PCR to quantify gene expression levels for TLR4 (A) and TLR9 (B). Results shown are the mean \pm SD of three to six replicates at each time point. Results shown are the mean \pm SD of six replicates at each time point. Relative fold changes (y-axis) for each gene were calculated by the Ct method and are relative to the gene expression in PBS injected skin tissue and each gene was normalized to pig HPRT1 for each time point. The letter code (b) depicts significant differences in gene expression between Emulsigen and PBS.

3.3.2. Induction of interferon induced genes and immune cell receptor gene expression by adjuvants

IFN- α , a viral response gene, was not induced in response to any of the adjuvants relative to PBS when administered to pig muscle but its expression was significantly repressed in response to Emulsigen after 48 hours (b;*) (Fig 7A). The protein coded for by IFNAR2 gene is a type I membrane protein (Domanski et al., 1995). IFNAR2 was induced less than 10-fold by Emulsigen at 48 and 96 hours (b; *) but no induction was observed in response to CpG or PCEP at any time points (Fig 7B). STAT2 is a transcriptional activator, which responds to cytokines such as IFN- α , and growth factors by being phosphorylated which triggers homo- or heterodimers and translocation to the nucleus (Holloway et al., 2014; Steen et al., 2013). CpG injected into pig skin induced expression of STAT2 at 6, 24 and 48 hours (a; *, ***, **) (Fig 7C) but no other adjuvant induced expression of this gene at any time points Awate *et al* (2012) showed that murine muscle injected with PCEP showed significant expression of STAT2 relative to tissue injected with PBS after 96 hours. Interferon regulatory factors (IRFs) are transcription factors that regulate expression of target cytokines such as interferon (IFN)- β and other innate immune response genes (Nehyba et al., 2009; Nehyba et al., 2002; Takaoka et al., 2008). IRF7 expression was repressed in pig skin in response to CpG relative to PBS (a; *) and relative to PCEP (f; *) after 1 hour but it was significantly induced in response to CpG relative to PBS after 24 and 48 hours (a; ***, ***) (Fig 7D) induced expression of IRF7 after 24, 48 and 96 hours (b; *, *, *, respectively) (Fig 7D). PCEP did not induce expression of IRF7 expression over time. The results of the present study are consistent with another study, which showed that, in response to injection of MF59 oil-in-water emulsion, CpG, and alum into murine muscle, IRF7, STAT1, and STAT2 gene expression were induced (Mosca et al., 2008).

IFN-stimulated genes IFIT2 and IFIT3 code for proteins which mediate protein-protein and protein-RNA interactions (Fensterl & Sen, 2011). IFIT2 gene was significantly induced in response to CpG for 24 and 48 hours (Fig 7E: a; **, *) and CpG induced IFIT2 expression relative to PCEP at 6, 24 and 48 hours, respectively (Fig 7E: f; *, **, *). IFIT3 gene was significantly induced in response to CpG (Fig 7F: a; ***, **) for 24 and 48 hours, respectively. CpG induced IFIT3 expression relative to PCEP at 48 hours (Fig 7F: f; (*)). Both genes showing peak expression relative to PBS of > 30 fold at 24 hours but both PCEP and Emulsigen did not significantly induce IFIT2 and IFIT3 gene expression over time in pigs. These results of the present study differ from the responses observed where PCEP was shown to increase IFIT2 and IFIT3 gene expression over time after intramuscular administration in mice (Awate et al., 2012).

MX1 is an Interferon-induced dynamin-like GTPase with antiviral activity against a wide range of RNA viruses and some DNA viruses (Jin et al., 1998; Verhelst et al., 2012). In the present study, CpG induced MX1 at 24 and 48 hours (a; ***, ***) (Fig 7G). In contrast, Awate et al (2012) showed that CpG and PCEP up-regulated expression of MX1 gene after injection into murine muscle but CpG induced a more rapid response and PCEP induced a >100 fold induction of MX1 after 48 hours (Awate et al., 2012). MX2 is known as an Interferon-induced dynamin-like GTPase with potent antiviral activity (Aebi et al., 1989). Others have shown that when CpG and Emulsigen were used as adjuvants in a foot and mouth disease vaccine, the vaccine triggered an increased transcription of MX1 when injected into pigs (Alves et al., 2009). In the present study, CpG significantly induced MX2 at 24 and 48 hours (a, ***, ***) (Fig 7H). Emulsigen induced the expression of MX2 after 48 hours (b; *). CpG significantly induced expression of MX1 (Fig 7G, f; *) and MX2 (Figure 7H: f;*) relative to PCEP. Awate *et al* (2012) showed that neither PCEP nor CpG induced expression of MX2 gene in murine muscle (Awate et al., 2012).

LGALS3 is an IFN-inducible gene that encodes a member of the galectin family of carbohydrate binding proteins. This protein plays a role in numerous cellular functions including apoptosis, innate immunity, cell adhesion and T-cell regulation (Chen et al., 2015). CpG induced LGALS3 gene expression after 24 hours and 48 hours (a; ****, **) and Emulsigen induced expression of this gene after 48 hours (b; *) (Fig 7I). In contrast to murine muscle, pig skin injected with PBS did not trigger a strong induction of LGALS3 gene expression over time (Awate et al., 2012). PSMB8 is an IFN-inducible gene whose corresponding protein is present in professional antigen presenting leucocytes and it is involved in the enzyme regulatory functions and proteosomal degradation. PSMB8 gene expression was induced in response to CpG at 24 hours (a; ***) (Fig 7J). PCEP and CpG were shown to up-regulate the expression of PSMB8 gene expression in mice muscle tissue at 48 and 96 hours (Awate et al., 2012). Our findings are consistent with the others that demonstrated that CpG was an efficient inducer of IFN- α related genes when injected intramuscularly.

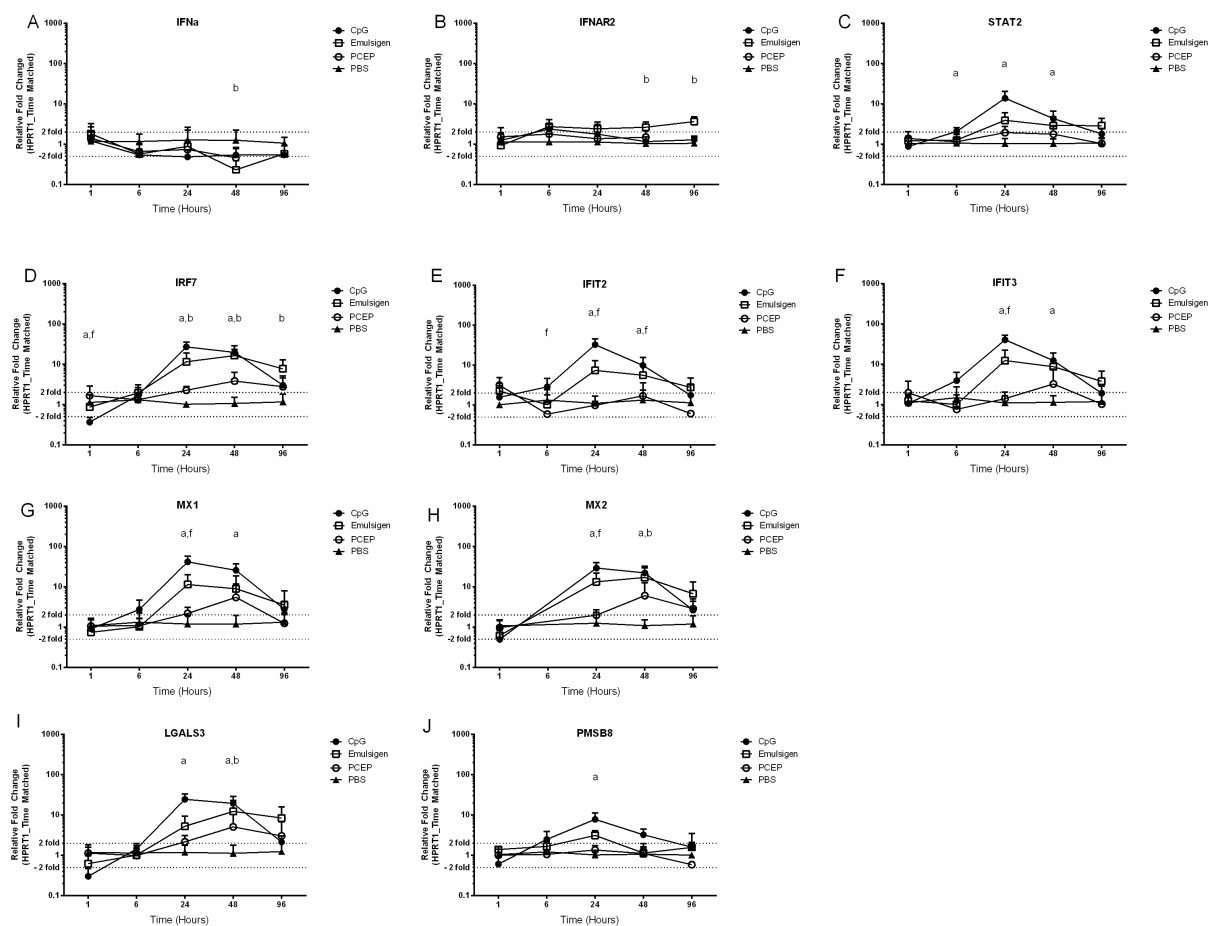


Figure 3.4 Induction of Interferon response genes in response to PCEP, CpG and Emulsigen at the site of intradermal administration in pigs. Skin punch biopsies were collected at 1, 6, 24, 48 and 96 hours and subjected to quantitative real-time PCR to quantify gene expression levels for IFN- α (A), IFNAR2 (B), STAT2 (C), IRF7 (D), IFIT2 (E), IFIT3 (F), MX1 (G), MX2 (H), LGALS3 (I) and PMSB8 (J). Results shown are the mean \pm SD of three to six replicates at each time point. Results shown are the mean \pm SD of six replicates at each time point. Relative fold changes (y-axis) for each gene were calculated by the Ct method and are relative to the gene expression in PBS injected skin tissue and each gene was normalized to pig HPRT1 for each time point. The letter codes depicting significant differences in gene expression between groups are as follows: (a) CpG to PBS, (b) Emulsigen to PBS, (c) PCEP to PBS, and (f) CpG to PCEP.

Figure 8 shows differential gene expression in response to adjuvants alone relative to PBS time matched controls. Expression data >2 are green and >10 are dark green. Gene expression less than 0.6 (-1.6 fold) relative to PBS are red and gene expression less than 0.4 (-2.5 fold) are dark red. Expression between 0.6 and 2 fold are yellow. With the exception of IL-17 which is induced at 1 hour (green) and then repressed in response to CpG (red) at later time points, the majority of genes tested showed peak gene induction or repression in response to CpG at 24 hours. The majority of genes showed induction in response to Emulsigen with expression peaking at 24 hours after stimulation. The response was not immediate, as the majority of genes were not induced after 1 or 6 hours. Approximately $\frac{1}{3}$ of the genes (such as chemokines and IL-6, IL-10, and IL-13) showed a > 2 fold induction of expression within 1 hour after exposure to PCEP with approximately $\frac{1}{4}$ of the genes showing induced expression over time with IL-17 gene expression being the exception. Finally, we observed that CpG, and to a much lesser extent, Emulsigen, were inducers of IFN response genes (IFIT2, IFIT3, MX1, MX2, IRF7, LGALS3, PMSB8, and STAT2) but IFN α itself was repressed and IRNAR2 was induced at later time points in response to Emulsigen. The results of the present study suggest that differences exist in the mechanisms mediating the adjuvant activities of PCEP, Emulsigen and CpG. Most genes were not stimulated or repressed in response to time matched average response to saline. Further studies are required to confirm whether the PCEP, Emulsigen or CpG trigger recruitment of innate immune cells at the site of injection or draining lymph nodes and whether they trigger differences in expression of local cytokines and IFN response genes. Understanding the mechanisms of action of adjuvants will provide critical information on how innate immunity influences the development of adaptive immunity in response to PCEP. Such knowledge will facilitate the rational development of new vaccine adjuvants.

	CpG					Emulsigen					PCEP					PBS				
	1 Hour	6 Hours	24 Hours	48 Hours	96 Hours	1 Hour	6 Hours	24 Hours	48 Hours	96 Hours	1 Hour	6 Hours	24 Hours	48 Hours	96 Hours	1 Hour	6 Hours	24 Hours	48 Hours	96 Hours
CCL2	0.78		9.17	5.21	1.91	0.78		24.77	13.51	10.75	3.64		5.37	5.96	7.44	1.30		1.32	1.21	1.27
CCL5	1.30		8.11	6.24	6.59	2.14		20.31	9.72	32.63	2.62		3.03	1.72	5.54	1.05		1.28	1.19	1.32
CXCL10	1.39	28.08	224.77	82.55	4.39	0.76	0.33	22.39	9.46	4.10	1.25	0.74	1.75	4.87		1.29	1.69	1.10	1.22	1.54
TNFA	0.96	2.53	2.77	1.97	1.15	1.02	2.77	6.96	4.83	3.45	5.24	1.67	1.85	1.64	1.90	1.24	1.03	1.06	1.09	1.03
NFKB	0.82	1.14	1.17	1.20	1.54	1.33	1.57	0.90	0.57	1.03	1.38	1.22	1.38	0.87	0.94	1.07	1.07	1.11	1.02	1.07
IL6	1.15	12.64	6.09	4.80	1.80	3.47	71.68	18.77	14.18	8.77	11.66	14.24	7.09	25.49	73.71	1.24	1.31	1.21	1.14	1.03
IL10	1.62	0.94	7.49	3.86	5.85	2.12	1.06	1.79	1.34	12.37	3.20	1.40	2.78	2.16	3.35	1.69	1.29	1.17	1.16	1.89
IL13	0.61	1.05	0.44	0.82	0.73	2.47	2.39	2.96	1.43	3.22	4.83	2.84	3.00	4.42		1.72	1.15	1.06	1.25	1.21
IL17	23.16	0.43	0.08	0.17		9.38	0.77	0.90	1.52		2.30	0.44	0.42	3.46		1.23	1.39	1.54	2.49	
TLR4	1.27	1.12	1.14	1.32	1.15	2.50	2.48	1.78	1.06	1.34	1.67	1.98	1.66	1.25	1.20	1.10	1.20	1.04	1.07	1.07
TLR9	1.15	1.25	2.31	4.16		1.56	5.67	7.66	10.99		2.28	1.70	3.97	5.87		1.11	1.10	1.23	1.08	
IFNA	1.21	0.54	0.44	0.54	0.55	1.82	0.57	0.88	0.43	0.58	1.42	0.66	0.74	0.46		1.18	1.17	1.27	1.26	1.06
IFNAR2	1.25	2.36	1.77	1.16	1.28	0.92	2.74	2.40	2.62	3.63	1.52	1.77	1.36	1.48		1.14	1.14	1.12	1.03	1.05
STAT2	0.88	2.06	13.77	4.31	1.79	1.22	1.26	3.90	2.90	2.87	1.38	1.14	1.93	1.77	1.03	1.05	1.05	1.04	1.04	1.06
IRF7	0.57	1.69	26.85	19.70	3.02	0.86	1.94	11.53	16.37	7.69	1.66	1.32	2.26	3.82	2.78	1.13	1.32	1.02	1.07	1.16
IFIT2	1.57	2.86	32.64	9.89	1.76	2.25	1.02	7.37	5.59	2.78	3.15	0.59	0.98	1.70	0.61	1.02	1.33	1.11	1.33	1.14
IFIT3	1.06	3.93	40.37	12.37	1.91	1.24	1.01	12.39	8.69	3.77	1.96	0.76	1.40	3.23	1.03	1.11	1.48	1.10	1.13	1.19
MX1	0.91	2.73	41.38	25.58	2.77	0.74	1.02	11.34	8.92	3.54	1.06	1.08	2.17	5.45	1.24	1.09	1.32	1.19	1.18	1.33
MX2	0.80		29.08	22.02	2.63	0.62		13.31	16.96	6.70	0.97		1.97	6.02	2.84	1.06		1.24	1.08	1.19
LGALS	0.33	1.45	24.66	19.47	2.15	0.63	1.02	5.28	12.35	8.45	1.14	0.99	2.17	5.10	3.04	1.18	1.15	1.19	1.13	1.24
PMSB8	0.61	2.49	7.86	3.25	1.60	1.40	1.67	3.14	1.15	1.58	1.02	1.06	1.36	1.12	0.59	1.04	1.23	1.03	1.07	1.02
MSR	1.07	1.16	1.47	0.73	1.20	1.17	1.06	3.56	0.84	4.12	1.53	2.01	2.68	0.85	1.30	1.20	1.12	1.03	1.15	1.17

Figure 3.5 Heat map of relative mRNA expression in pig skin induced by administration of CpG, Emulsigen, PCEP or PBS at the site of injection. Each column represents one time point and each row represents the relative fold change gene expression for each gene at that time point. The colour scheme for the relative gene expression is as follows: <0.5 is dark red, between 0.4 and 0.6 is red, between 0.61 and 1.8 is yellow, between 1.8 and 10 is green, > 10 is dark green. Gene names are listed on the left of panel and time points and treatment are indicated across the top of each panel.

Chapter 4. Innate immune response profiles in pigs injected with vaccine adjuvants polydi(sodium carboxylatoethylphenoxy)phosphazene (PCEP) and Emulsigen

(Veterinary Immunology Immunopathology, 2019: 209, 7-16)

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Relationship of this study to the dissertation

In chapter 3, we have shown that PCEP is a strong modulator of immune response genes. In this chapter we evaluate whether gene expression translate to protein transcription by accessing local production of cytokines after intradermal injection in pigs and whether cytokines induce recruitment of immune cells at the site of injection. In this study, we examined PCEP-induced immune cell recruitment at the injection site and changes in cell composition in the draining lymph nodes. Additionally, we determined the cytokine responses at the site of injection. Taken together, PCEP induced significant production of interleukin IL-1 β , and IL-13 at the site of injection which leads to recruitment of distinct immune cells to the site of injection site suggesting that PCEP stimulated early innate immune responses at the injection site by creating an immunocompetent environment that may contribute to increased immunogenicity of the co-administered antigens.

In this manuscript my role was conception of ideas, design, planning and conducting experiments, data analysis and interpretation. I also prepared draft of the manuscript and revised it for intellectual content; and final approval of the version to be published. Additionally, I responded to reviewer's comments as appropriate.

4.1 Introduction

Effective adjuvants enhance the immunogenicity of highly purified or recombinant antigens but despite being used for decades in vaccines, MOA of many adjuvants remain unknown. This lack of clarity regarding adjuvant MOA is one of the factors that has limited development and approval of new adjuvants for human use (Calabro et al., 2011). Vaccination with antigen alone often triggers little or no specific immune responses unless formulated with an adjuvant that activates the innate immune responses (Calabro et al., 2011; Levitz & Golenbock, 2012; Liang & Loré, 2016; McKee et al., 2009; Pasquale et al., 2015; Schijns, 2002; Wang & Singh, 2011). Studies in mice indicate that vaccine adjuvants enhance the immunogenicity of the antigen by activating early innate immune responses and promoting a strong immunostimulatory environment at the site of injection (Gupta & Chaphalkar, 2015; Kanzler et al., 2007; Milligan, 2014).

We previously reported that PCEP, a high-molecular weight, water-soluble polymer, enhanced long lasting immune responses when co-administered with a variety of viral and bacterial antigens in mice, pigs and cattle (Awate et al., 2012; Eng et al., 2010; Magiri et al., 2018b; McNeal et al., 1999; Mutwiri et al., 2008; Mutwiri et al., 2007). We recently reported on PCEP MOA in a series of studies. In mice similar to other adjuvants, PCEP induced recruitment of immune cells to the site of injection and promoted transport of antigen to the draining lymph

node (Awate et al., 2014; Dupuis et al., 2001). The cells recruited following intramuscular (i.m.) injection of PCEP to the site of injection and the draining lymph nodes in mice constituted largely of neutrophils but also macrophages, CD4⁺ T cells, CD8⁺ T cells and CD19⁺ B cells, monocytes and DCs (Awate et al., 2014). Further, PCEP promoted cytokine and chemokine production as well as regulation of a number of ‘adjuvant core response genes’ at the intramuscular injection site in mice and pigs (Awate et al., 2012; Magiri et al., 2016; Mutwiri et al., 2008). Specifically, studies with mice and pigs revealed species-specific differences in polyphosphazenes induced stimulation of innate immune responses (S. Awate et al., 2012; Magiri et al., 2016). Intramuscular injection of PCEP induced time-dependent changes in the gene expression of many “adjuvant core response genes” (Mosca et al., 2008a) such as chemokine genes CCL-2, CCL-4, CCL-5, CCL-12 and CXCL-10 in mice (Awate et al., 2012) and CCL2 and CXCL10 (but not CCL- 5) in pigs (Magiri et al., 2016). Major transcription factor NF- κ B gene and the inflammatory cytokine TNF- α genes were up-regulated in response to PCEP in mice (Awate et al., 2012) but not in pigs (Magiri et al., 2016). Additionally, in pigs, PCEP induced IL-6 gene expression but not IL-10, IL-17 or IFN- α (Magiri et al 2016). PCEP injection in mice increased the expression of TLR4 and TLR9 at the site of injection (Awate et al., 2012) whereas PCEP did not induce significant expression any of the TLR genes in pigs suggesting differences in activation of immune responses in different animal species (Magiri et al., 2016). However, across species, PCEP may modulate antigen-specific immune responses by activating early innate immune responses and promoting a strong immunostimulatory environment at the site of injection. By promoting induction of transient innate immune responses, adjuvants may promote antigen-specific immunity.

Very little is known about MOA of adjuvants in pigs. We recently reported that PCEP induced the expression of chemokines and proinflammatory cytokines genes when injected

intrademally in pigs (Magiri et al., 2016) suggesting that PCEP may promote recruitment of immune cells at the site of injection. Further unlike what was observed in mice, PCEP did not induce significant expression any of the TLR genes in pigs suggesting species specific differences in activation of innate immune responses (Magiri et al., 2016). We hypothesize that PCEP induces an innate immune response at injection sites and thus creates an immune microenvironment to facilitate vaccine triggered adaptive immunity. In the current study, we investigated whether PCEP activates cytokine production and recruitment of immune cells in pigs by evaluating changes at the site of injection and the draining lymph nodes. A known commercial adjuvant for pig vaccines, Emulsigen was included for comparative purposes.

4.2 MATERIALS AND METHODS

4.2.1 Animal experiments

The animal experiments were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhere to the Canadian Council on Animal Care guidelines for humane animal use. Groups consisted of 2 control groups (naïve, unimmunized animals and pigs injected with PBS) and 2 treatment groups (pigs injected with PCEP or emulsigen). The animals were 3-4 four weeks old commercial cross breed pigs (20 animals per group with n=5 animals euthanized at 4 specific time points (Days 1, 4, 7 and 14)) were administered intradermal (I.D.) injection on the neck region left and right side (250 µl per side) with sterile phosphate-buffered saline (PBS), 500 µg PCEP, or 20% Emulsigen and an additional group without any injection as naive. The body temperature, and clinical observations & score for local reactions in all groups at both injections sites were taken through out the study period. The local reaction scores microscopically were

from 0 - 3 with 0 = normal, 1 = minor, 2 = moderate & 3 = severe. Additionally, markings of injection site were reapplied every day so that they can be visible at all time point. An 8 mm skin punch plug biopsy and the corresponding draining lymph nodes were collected from five animals per group at 1, 4, 7, and 14 days post injection. Biopsies were treated with 10% formalin for histopathology or incubated with RPMI supplemented with 5% FBS for tissue homogenisation and subsequent cytometric bead assay. Fifty microliters of peripheral blood was collected into serum separation tubes (SST) for cytokines cytometric bead assay at each time point in the serum.

4.2.2 Adjuvants preparation

PCEP was synthesized by the Idaho National Laboratory (Idaho Falls, ID, USA) using methods previously described in (Andrianov et al., 2004; Mutwiri et al., 2007b) and its endotoxin level was determined to be less than 0.034 ng/ml as assessed by the Limulus Amebocyte Lysate assay (Biowhittaker, Walkersville, MD, USA). PCEP and an “oil-in-water” formulation (Emulsigen) (MVP Laboratories, OM, USA) were dissolved in endotoxin-free, sterile PBS, pH 7.4 (Life Technologies, Carlsbad, CA, USA) prior to injection.

4.2.3 Histopathology preparation

Skin punch biopsy and the draining lymph nodes tissues were prepared for histopathological evaluations as follows: Tissues were fixed with 10% formalin, then incubated for 24 hour in 70% ethanol, 100 % ethanol and finally 100% xylol. Tissues were then embedded in paraffin block for easier sectioning in the microtome and sliced to 3 to 5 μ m thickness. Slides were stained using standard Haematoxylin-eosin method following the standard operating procedures. Histopathological changes of the skins that were evaluated included granuloma

formation, lymphocytes infiltration, epithelial necrosis, and suppurative inflammation. Histopathological changes in the draining lymph nodes included granuloma formation and suppurative inflammation. Scoring was performed by a pathologist blinded to the treatment and the scoring was as follows: A) No pathological changes = 0, B) Patchy pathological changes = 1, C) Moderate pathological changes = 2, D) Severe pathological changes = 3 as described previously by (Magiri et al., 2018a; Mikalsen et al., 2012).

4.2.4 Cytometric bead assay

Cytokine concentrations were assayed in sera as well as from tissue homogenates from the injection site and the draining lymph nodes. Skin punch biopsy and draining lymph nodes were homogenized in 1 ml PBS with protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany) with 2.3 mm Zirconia silica microbeads (Biospec Products Inc., Bartlesville, OK, USA) in a mini-beadbeaterTM (Biospec Products, Inc.) via six 10 second pulses interspersed with cooling at 4°C. The homogenates were centrifuged at 20,000 ×g for 10 min at 4°C, and supernatants stored at -20°C before analysis. Cytokine levels were measured by cytometric bead assay (BioRad) according to the manufacturer's recommendations.

Bioplex cytokine assays: Bioplex bead coupling was performed as per the manufacturer's instructions. The reagents used are described in table 1. BioPlex cytokine assays antibodies against porcine IFN- α , IFN- γ , IL-1- β , IL-6, IL-8, IL-10, IL-12, IL-13, and IL-17A were coupled to BioRad multiplex assay microsphere beads using the BioRad BioPlex following manufacturer's instructions. Sera and tissue homogenates were tested for IFN- α , IFN- γ , IL-1- β , IL-6, IL-8, IL-10, IL-12, IL-13, and IL-17A secretion. The multiplex assay was carried out in a 96 well Greiner Bio-One Fluotrac 200 96Fblack (VWR, #82050-754), which allows washing and retention of the Luminex beads. The bead sets conjugated with all the cytokine to be analyzed

were vortexed for 30 seconds followed by sonication for another 30 seconds to ensure total bead dispersal. The bead density was adjusted to 1200 beads per μl in PBSA (1x PBS + 1% BSA (Sigma-Aldrich) + 0.05 % sodium azide (Sigma-Aldrich), pH 7.4) and 1 μl of each bead set was added to 49 μl of the PBSA + 1% New Zealand Pig Serum (Sigma-Aldrich P3484) +0.05 % sodium azide (Sigma-Aldrich) which was then added to each well. Plates were washed using the Bio-Plex Pro II Wash Station (Bio-Rad) to allow soaking for 20 s then washing with 100 μl PBSA. The porcine cytokine protein standards were added to the wells at 50 μl per well at a final concentration as described in table 1 below and the serum (prediluted 1:4) and tissue homogenate (injection sites and draining lymph nodes; prediluted 1:2) were added to the wells at 50 μl per well. After sealing the plates with plate sealer (Thermo Fisher Scientific, #232702), the plates were agitated at 800 rpm for 1 hour at room temperature. After 1 hour incubation with serum, the plates were washed using the Bio-Plex Pro II Wash Station (Bio-Rad; soak 30 s, wash with 150 μl PBS plus 1% Tween-20). A 50 μl volume of biotin cocktail consisting of biotinylated porcine antibodies (as described in table 1) was added to each well. The plates were again sealed, covered and agitated at 800 rpm for 30 minutes at room temperature then washed again as described above. A 50 μl of Streptavidin RPE (Cedarlane PJRS20; diluted to 5 $\mu\text{g}/\text{ml}$) was added to each well. The plate was again sealed, covered and agitated at 800 rpm for 30 minutes at room temperature and washed as indicated above. A 100 μl of 1xTris-EDTA (TE buffer-10 mM Tris, bring to pH 8.0 with HCl and 1 mM EDTA) was added to each well and then the plate was vortexed for 5 minutes before reading on the Luminex100 xMAP™ instrument following the manufacturer's instructions). The fluorescence on the beads was read on a BioRad BioPlex 200 reader (60 μl volume, 50 beads per region).

Table 4.1 List of antibodies, bead, detection (secondary antibodies) and suppliers

Cytokine standards		Standard concentration range
Cytokine	Supplier/ Catalog number	(pg/ml)
rPorc IFN alpha	Genentech	200-1 pg/mL
rPorc IFN gamma	CG	2000-8 pg/mL
rPorc IL-1 β	R&D 681PI010 (10 μ g)	5000-20 pg/mL
rPorc IL-6	R&D 686PI025 (25 μ g)	5000-20 pg/mL
rPorc IL-8	Kingfisher RP0109S-005 (5 μ g)	200-1 pg/mL
rPorc IL-10	Invitrogen PSC0104 (10 μ g)	5000-20 pg/mL
rPorc IL-12	R&D 912PL025 (25 μ g)	5000-20 pg/mL
rPorc IL-13	Kingfisher RP0007S-005 (5 μ g)	5000-20 pg/mL
rPorc IL-17A	Kingfisher RP0128S-005 (5 μ g)	2000-8 pg/mL
Coating antibodies to Coupled beads		
Cytokine	Catalog number	Bead region (all from BioRad)
MAb anti IFN alpha	GeneTex GTX11408	45 (BioRad MC10045-01)
MAb anti porc IFN gamma	Fisher PIMP700	43 (BioRad MC10043-01)
MAb anti porc IL-1b/IF2	R&D MAB6811	26 (BioRad MC10026-01)
Goat anti porcine IL-6	R&D AF686	65 (BioRad MC10065-01)
MAb anti sheep IL8 (86.9% homology)	AbD Serotec MCA1660	27 (BioRad MC10027-01)
MAb anti swine IL10	Invitrogen ASC0104	28 (BioRad MC10028-01)
Porc IL-12	MAb anti porc IL12	36 (BioRad MC10036-01)
Goat anti swine IL-13	Kingfisher PB0094S-100	52 (BioRad MC10052-01)

Rabbit anti porcine IL-17A	Kingfisher KP0498S-100	62 (BioRad MC10062-01)
Detection antibodies (all biotinylated)		
Cytokine	Catalog number	Desired starting conc (ug/ml)
MAB anti pig IFN alpha (biotin in house)	R&D 27105-1	1/5000
Rabbit anti porc IFN gamma (biotin in house)	Fisher PIPP700	1/400
Goat anti porc IL-1b/IF2 biotin	R&D BAF681 (50 µg)	0.5 µg/mL
Goat anti porcine IL-6 biotin	R&D BAF686 (50 µg)	0.5 µg/mL
MAB anti porc CXCL8/IL8 (biotin in house)	R&D MAB5351 (500 µg)	1/500
MAB anti swine IL10 biotin	Invitrogen ASC9109	0.5 µg/mL for cell sups, 1 µg/mL for sera
MAB anti porc IL12/IL23 p40 biotin	R&D BAM9122	0.5 µg/mL
Goat anti swine IL-13 biotin	Kingfisher PBB0096S-050	0.5 µg/mL
Rabbit anti porcine IL-17A biotin	Kingfisher KPB0499S-050	0.5 µg/mL

4.2.5 Data analysis

Statistical analyses were performed using Graph-Pad Prism 7 software (GraphPad Software, San Diego, CA, USA). Differences between groups were identified using a non-parametric Kruskal-Wallis ANOVA test where Dunn's multiple comparisons test was used post-hoc to identify statistically significant differences between the two adjuvants and saline control

relative to each time point. Differences were considered statistically significant at $p < 0.05$, $p < 0.01$, $p < 0.001$ which are stated in the text.

4.3 Results

4.3.1 Histopathological changes of the skin and draining lymph nodes after i.d. Injection with PCEP and Emulsigen

Pigs were injected i.d. with PBS, PCEP or Emulsigen. Body temperature was taken and injection sites were examined for gross changes. In all the animals, body temperatures remained normal throughout the study period suggesting that the adjuvants did not induce a systemic response (data not shown). The local inflammatory reaction did not induce gross ulceration at the site of injection as observed macroscopically (data not shown).

Sites of injection and draining lymph nodes were excised and evaluated for evidence of an inflammatory response and cytokine production. We evaluated changes in histopathology including granuloma formation (microscopic aggregation of macrophages transformed into epithelium like cells surrounded by a collar of mononuclear leukocytes, principally lymphocytes, and plasma cells), lymphocytes infiltration (infiltration of tissue by T and B lymphocytes), epithelial necrosis (death of cells and living tissues), and suppurative inflammation (forms as a result of the action of polymorphonuclear leukocytes e.g. neutrophils) (Fig 9). Representative photomicrographs show a typical cellular infiltrate observed in the skin at the site of injection (Fig 9 left) and the draining lymph nodes (Fig 9 right) on day 14 post injection. The top panel shows normal skin (Fig 9A) or draining lymph nodes (Fig 9B) after injection with saline at 200x magnification. In the skin PCEP (Fig 9C) induced multifocal to coalescing inflammation and a mixture of macrophages (blue arrow), lymphocytes (yellow arrow) and some neutrophils (red

arrow, centre image) with many necrotic debris in the inflammation (blue arrows). In the draining lymph nodes, PCEP (Fig 9D) induced multifocal infiltration of macrophages, with multinucleated giant cells (blue arrow) and sparse neutrophils (red arrow). In the skin, Emulsigen (Fig 1E) induced diffuse infiltration of inflammatory cells, composed of macrophages and lymphocytes and small granuloma. In the draining lymph nodes, Emulsigen (Fig 9F) induced coalescing areas of granulomatous inflammation (blue arrows) and inflammation is composed of mostly macrophages. Collectively, these data show the ability of adjuvants to promote local inflammation, which may be important for the initiation of the innate and acquired immune response.

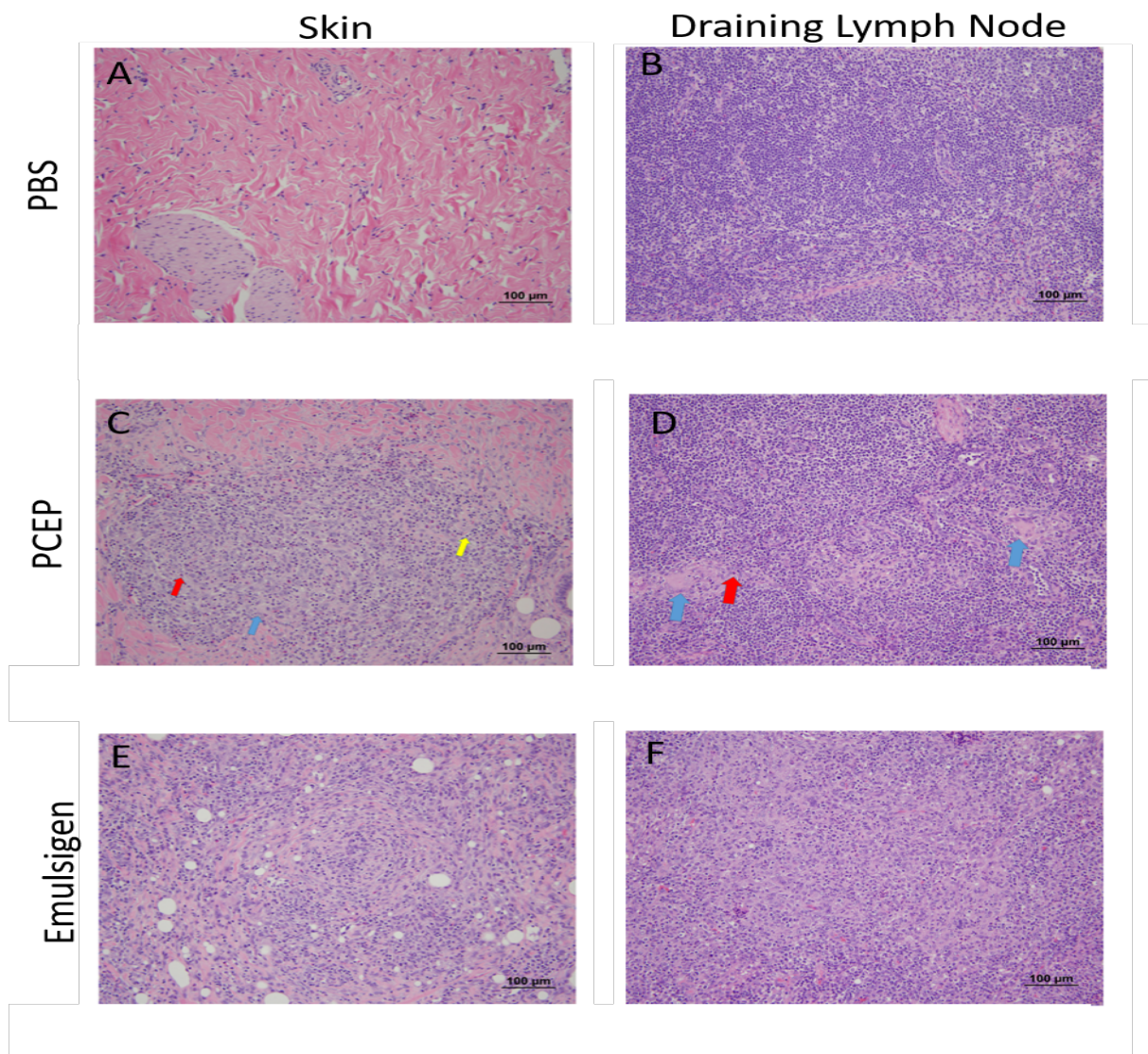


Figure 4.1 Representative images of the cellular infiltrate at the injection site and lymph nodes draining of the site injected with either saline, PCEP or Emulsigen adjuvants at a magnification of X200. Top panel shows normal skin injected with saline at the skin site of injection (left) and the draining lymph nodes (right). On the skin PCEP (middle left panel) induced multifocal to coalescing inflammation and a mixture of macrophages (blue arrow), lymphocytes (yellow arrow) and some neutrophils (red arrow, centre image) with many necrotic debris in the inflammation (blue arrows) while on the draining lymphnodes PCEP (middle right panel) induced multifocal infiltration of macrophages, with multinucleated giant cells (blue arrow) and sparse neutrophils (red arrow). On the skin site of injection, Emulsigen (lower left panel) induced diffuse infiltration of inflammatory cells (left), composed of macrophages and lymphocytes and small granuloma while on the draining lymph nodes, Emulsigen (lower right panel) induced coalescing areas of granulomatous inflammation (blue arrows) and inflammation is composed of mostly macrophages.

Histopathological scoring was performed by a pathologist who was blinded to the sample identification. At the site of injection, adjuvants induced significant granuloma formation, lymphocytes infiltration, suppurative inflammation and necrosis compared to the skin of pigs injected with saline as a control. Interestingly, no granulomas were observed until after 14 days with pigs injected with Emulsigen ($p < 0.05$) and PCEP ($p < 0.01$) showing significant granuloma formation at the injection site relative to time-matched saline control animals (Fig 10A). Further, pigs injected with PCEP showed significant lymphocytic infiltration 4 ($p < 0.05$) and 14 days ($p < 0.01$) post administration relative to the saline control pigs (Fig 10B). Pigs injected with Emulsigen showed significant lymphocytic infiltration 14 days ($p < 0.05$) post administration (Fig 10B). PCEP and Emulsigen induced significant tissue necrosis 1, 4 and 7 days post administration ($p < 0.01$, $p < 0.01$, $p < 0.01$ for PCEP); $p < 0.05$, $p < 0.05$, $p < 0.01$ for Emulsigen, respectively) at the site of injection relative to time-matched saline controls (Fig 10C). However, signs of necrosis were resolved by 14 days suggesting rapid resolution of the tissue reaction. PCEP induced significant suppurative inflammation with predominant eosinophil and neutrophil infiltration for 1 ($p < 0.01$), 4 ($p < 0.05$), 7 ($p < 0.01$) and 14 ($p < 0.01$) days post injection relative to the saline controls (Fig 10D). Emulsigen induced significant suppurative inflammation on days 1 ($p < 0.05$), 4 ($p < 0.01$) and 7 ($p < 0.05$) post injection (Fig 10D).

In the draining lymph nodes, Emulsigen induced significant granuloma formation at day 4 ($p < 0.05$) and day 14 ($p < 0.05$) and PCEP induced significant granuloma formation at 7 days ($p < 0.05$) post inoculation relative to the saline control animals (Fig 10E). Finally, PCEP induced significant suppurative inflammation in the draining lymph nodes 1 day post injection relative to lymph nodes from saline control animals ($p < 0.05$) but the response was not significantly different from controls at other time points suggesting rapid resolution of the inflammation (Figure 10F).

PCEP and Emulsigen induced histopathological changes in skin and draining lymph nodes relative to pigs with saline as a control.

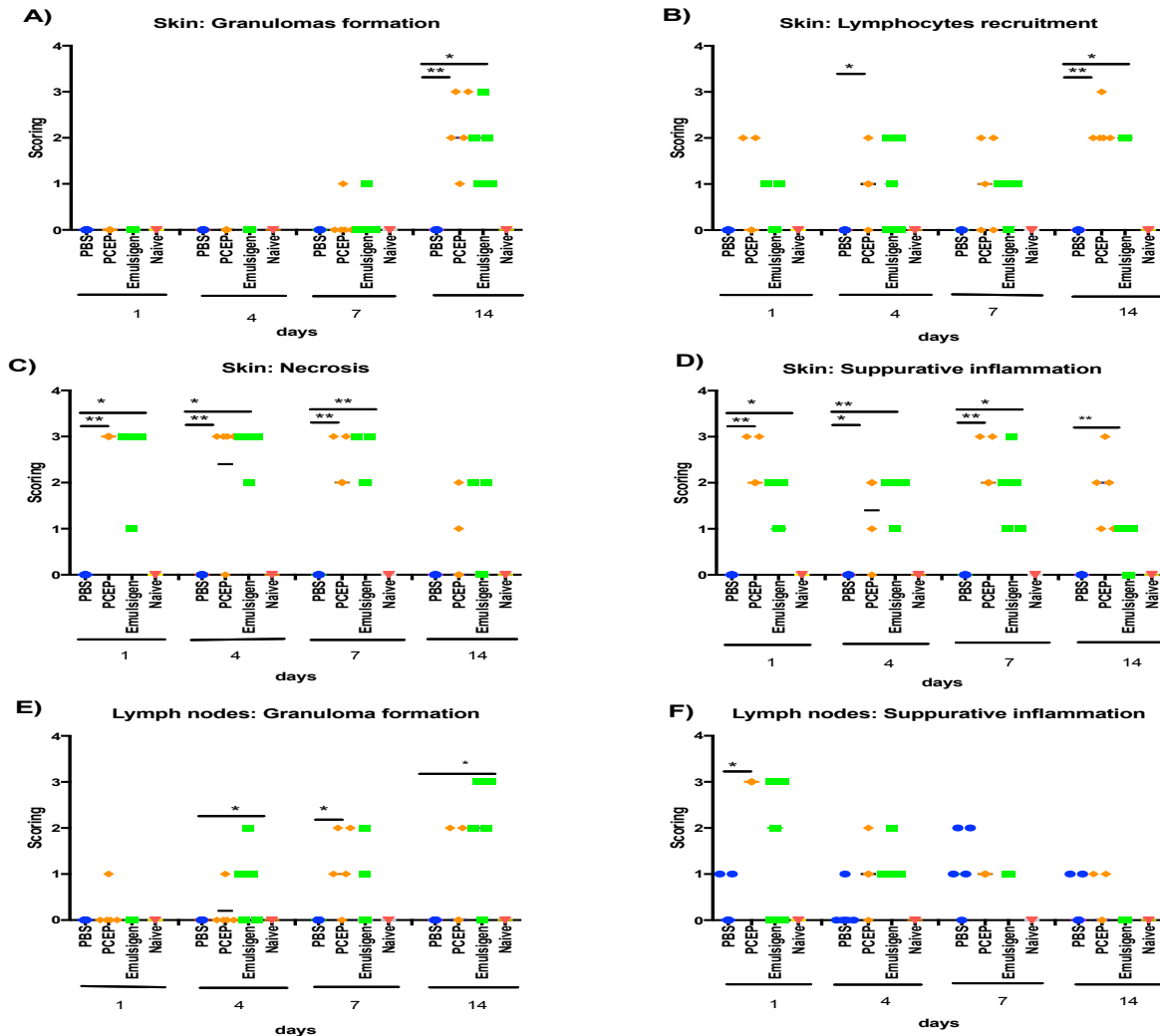


Figure 4.2 The histopathological changes of in the skin and draining lymph nodes in response to intradermal injection of PCEP or Emulsigen. The site of injection at the skin and the draining lymph nodes were collected from the draining lymph nodes at 1, 4, 7, and 14 days post injection. The lines indicate the median value of five replicates at each time point. All tissues are compared to time-matched skin or lymph nodes tissues from saline-injected control animals. Histopathological changes in the skin included assessment of granuloma formation, lymphocyte recruitment, necrosis, and signs of inflammation whereas histopathology of draining lymph nodes included assessment of granuloma formation and suppurative inflammation. Scoring was performed by blinded pathologist.

4.3.2 Cytokine profile in the pig skin, draining lymph nodes and peripheral blood after i.d. injection with PCEP and Emulsigen.

Punch biopsies were obtained from the site of intradermal injection was collected after 1, 4, 7 and 14 days in pigs inoculated with saline, PCEP and Emulsigen. Cytometric bead cytokine analysis was performed on homogenates of the biopsies. Emulsigen triggered significantly increased production of IL-6 ($p<0.05$; Fig 11D) and IL1 β ($p<0.05$; Fig 11C) after 4 days and of IL-12 ($p<0.05$; Fig 11G) after 7 days. PCEP triggered increased local production of IL1 β ($p<0.01$; Fig 11C) and IL-13 ($p<0.01$; Fig 11H) after 4 days relative to the control skin tissue. These data indicate that despite the evidence of microscopic histopathological evidence at the site of injection in response to PCEP and Emulsigen, only acute inflammatory cytokines IL-6 (Emulsigen only) and IL1 β (PCEP and Emulsigen) were transiently induced at the site of injection in pigs 4 days post injection. Neither of the adjuvants triggered local production of IFN- α (Fig 11A), IFN- γ (Fig 11B), IL-8 (Fig 11E), IL-10 (Fig 11F) or IL-17 (Fig 11I) relative to the biopsies from the saline injection sites.

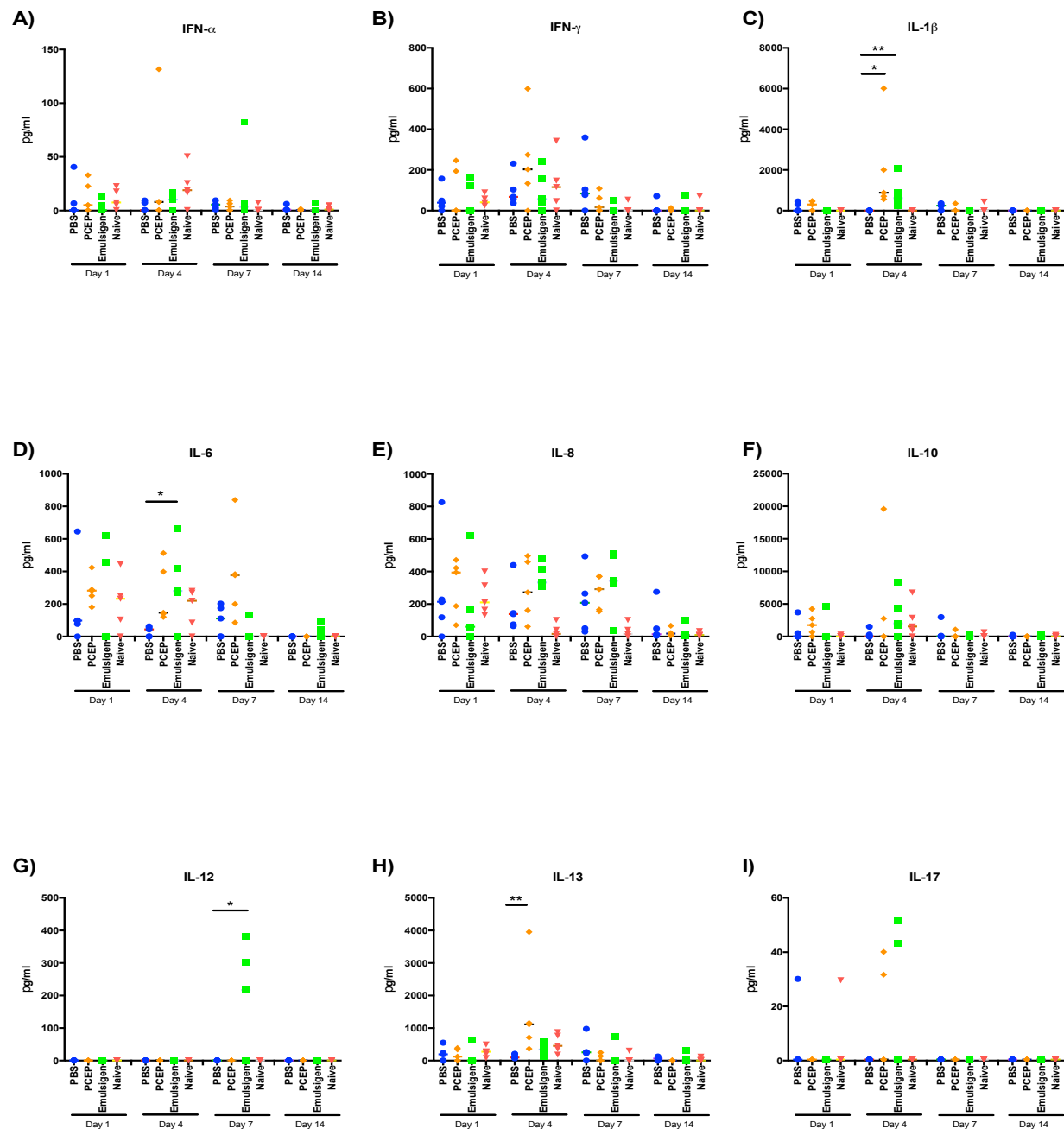


Figure 4.3 Cytokine responses in the skin at the injection site. The injection site were collected from one of the sites at 1, 4, 7, and 14 days post injection incubated with RPMI supplemented with 5% FBS for tissue homogenisation in PBS and cytometric bead assay using Bioplex. Results shown are the mean \pm SD *five* replicates at each time point compared to PBS

When cytokine analysis was assessed at the draining lymph nodes, PCEP induced secretion of interleukin IL-1 β ($p < 0.01$; Fig 12A) and IL-6 ($p < 0.01$; Fig 12B) on day 1 post injection but this elevation of these returned to baseline levels by day 4 post injection (figure 11). Further, neither PCEP nor Emulsigen induced secretion of IFN- α (Data not shown), IFN- γ (data not shown), IL-8 (Fig 12C), IL-10 (Fig 4D), IL-12 (Fig 12E), IL-13 (Fig 12F) or IL-17 (Fig 12G) at the draining lymph nodes over time.

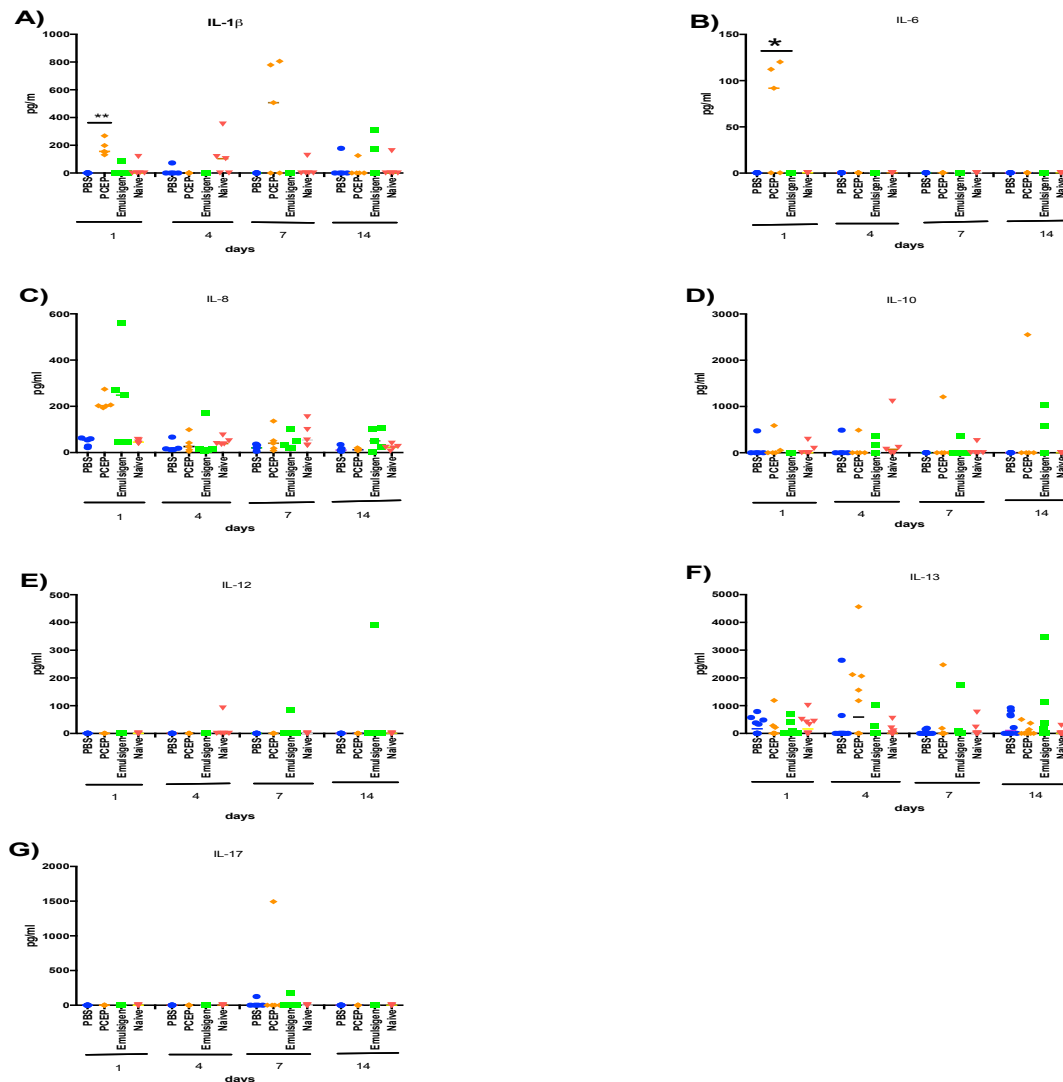


Figure 4.4 Cytokine responses in lymph nodes draining the injection site. The lymph nodes were collected from one of the sites at 1, 4, 7, and 14 days post injection incubated with RPMI supplemented with 5%

Finally, serum cytokine profiles were assessed over time in pigs i.d. injected with PCEP, Emulsigen and saline. None of the immunostimulants resulted in elevated production of serum IFN- α , IL-8, IFN- γ , IL-17, IL-6, IL1 β , IL-10, IL-12 or IL-13 (Data not shown) indicating that the effects of the PCEP and Emulsigen as immunostimulants were observed locally but not systemically.

4.4 Discussion

Vaccination continues to be a very important public health tool in the control of infectious diseases in the world. The main goal of vaccination is to stimulate potent immunological responses which promotes protection against specific pathogens (Kaeche et al., 2002). Immunostimulatory adjuvants can direct innate and adaptive immune responses by promoting DC maturation and the concomitant release of pro-inflammatory cytokines (Liang & Loré, 2016; Mizumoto et al., 2005). Together, these effects significantly impact the overall efficacy of vaccines when immunostimulatory adjuvants are included (Coler et al., 2011). Our study attempts to elucidate the impact that immunostimulants PCEP and Emulsigen have at sites of injection and draining lymph nodes in pigs over a two week period. The impact on the innate immune and inflammatory response is instructive of how the adjuvants will influence the immune response when used as part of a vaccine.

We selected the i.d. route of administration for our analysis because we showed it was more superior than i.m and it is a common route for administration of vaccines in livestock (Zhang et al., 2017, Magiri et al., 2018a) and recently, we showed that i.d. administration of a H1N1 vaccine which included PCEP as adjuvant gave a superior immune responses compared to pigs immunized with the same vaccine but administered via the intramuscular route (Magiri et al., 2018b). Skin-associated lymphoid tissue consists mainly of Langerhans cells and dermal APCs which circulate between the skin and the lymph nodes (DeBenedictis et al., 2001; Ray & Gately, 1996). Some reports suggest that i.d. vaccines induce superior protective immune responses and required less antigen relative to vaccines administered i.m. or subcutaneously (Herbert et al., 1989; Itzchak et al., 1992; Magiri et al., 2018a). Overall, our results showed that PCEP and Emulsigen induced strong inflammatory response at the site of injection and the

draining lymph node, suggesting that inflammation may be critically important for the initiation of the innate immunity and the promotion of acquired immunity.

We investigated histopathological changes such as granuloma formation with presence of macrophages, lymphocytes infiltration with T and B cells, epithelial necrosis, and suppurative inflammation (characterised predominantly as neutrophils and eosinophils). Interestingly, no macroscopic changes were observed for either adjuvant but PCEP induced significant macrophages infiltration, lymphocyte infiltration, leucocyte infiltration and necrotic debris at the site of injection and the draining lymph nodes whereas Emulsigen induced diffuse infiltration of leucocytes, macrophages, and lymphocytes at both sites. PCEP-induced signs of necrosis were resolved after 14 days post injection of the immunostimulants. These results are consistent with results from other authors who have described adjuvants induce a local microscopic inflammatory reaction at an injection site (Calabro et al., 2011; Schijns, 2000) without gross macroscopic ulcerations (das Does Moreira et al., 2009). Early stages of inflammation can occur through the activation of pattern-recognition receptors on many cells including lymphocytes, granulocytes, and endothelial cells (Faure et al., 2001; Muzio et al., 2000). Tissue necrosis induced by adjuvants as in “danger signal” hypothesis advanced by (Matzinger, 1994), who proposed that apart from self/non-self discrimination against infection, danger signals from damaged cells can trigger activation of the immune system through activation of inflammasome leading to release of proinflammatory cytokine such as IL-1 β . Molecules associated with tissue damage such as uric acid, nucleotides, adenosine triphosphate (ATP), reactive oxygen intermediates, and cytokines are released at the injection site due to tissue damage (Shi et al., 2003). These non-infectious damage signals have now been named DAMPs to distinguish them from PAMPs. Particulate adjuvants cause local tissue damage and cell death at the injection site and release of DAMPs thereby activating inflammasome (Kool et al., 2008). In addition, many

adjuvants induce release of pro-inflammatory cytokines at the site of injection including PCEP (Awate et al., 2012; Calabro et al., 2011; Didierlaurent et al., 2009). These damage signals trigger non-specific activation of the innate immune system, subsequently stimulating adaptive immunity. Recently inflammasomes have been one of the most widely investigated topics due to their potential role in adjuvant activity.

The role of neutrophils in adjuvant activity is not completely understood but increased neutrophils at the site of injection may attract other immune cells due to chemokine release ultimately resulting in increased antigen uptake, processing and transport to the draining lymph nodes (Calabro et al., 2011; Morel et al., 2011). In agreement with the current findings, PCEP injection was shown to lead to robust local infiltration by T and B lymphocyte in mice (Awate et al., 2014). Studies in mice have shown that PCEP is a potent inducer of cell recruitment at the injection site with lymphocytes, neutrophils and macrophages being the most abundant cells, followed by monocytes and DCs in mice (Awate et al., 2014a).

Although the adjuvants PCEP and Emulsigen were able to influence the immune response, their mechanism of action that can differentiate the intensity or duration of the required immune response may not be conserved. Thus, we also evaluated the cytokine profile to clarify how early events relate to precise immune response to adjuvants. Many adjuvants induce release of pro-inflammatory cytokines at the site of injection in mice (Awate et al., 2012; Calabro et al., 2011; Didierlaurent et al., 2009). Other studies have shown the ability of oil-in-water emulsion AS03 co-localize with antigen to trigger colony-stimulating factor 3 (CSF3) and IL-6, and leukocyte- recruiting chemokines CCL2, CCL3, and CCL5 at the site of injection (Morel et al., 2011) and draining lymph nodes (Morel et al., 2011). Studies in mice and pigs showed that PCEP induced strong expression of adjuvant core response genes coding for multiple cytokines and chemokines and immunomodulatory proteins (Awate et al., 2012; Magiri et al., 2016). In the

current study, we observed that PCEP and Emulsigen induced secretion of proinflammatory cytokines acute inflammatory cytokines IL-1 β and IL-6 (Emulsigen only). IL-6 is a proinflammatory cytokine that is involved in the initiation of an immune response and it also promotes the differentiation of B cells (Helle et al., 1988; Hilbert et al., 1989; Houssiau et al., 1988). PCEP may therefore direct the immune response towards a Th2 type of adaptive response. IL1 β cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation and differentiation, and apoptosis. IL1 β cytokine is proteolytically processed to its active form by caspase 1 activating multiprotein inflammasomes which assemble in the cytoplasm of cells. Activated caspase-1 cleaves the pro-forms of the interleukin-1 cytokine family members leading to their activation and secretion of active form of IL1 β (Bennouna et al., 2003; Brereton et al., 2011; Gris et al., 2010; Latz, 2010). PCEP and Emulsigen-induced inflammasome activation may play an important role in activating innate immunity thus contributing to adjuvant activity in pigs. Results from *in vitro* experiments with murine cells indicate that PCEP stimulated significant production of the innate immune response cytokines IL-12 and IFN- γ (Garlapati et al., 2011; Mutwiri et al., 2008). Activated DCs produce cytokines such as IL-12 which stimulates development of T helper cells that produce IFN- γ and promote cell-mediated immunity (Th1 cells). These results in mice are in contrast to our findings in pigs wherein we observed transient induction of IL-6 and IL-1 β at draining lymph nodes. At the skin, PCEP induced transient expression of IL-1 β as well as IL-13, an anti-inflammatory cytokine, and a mediator of allergic inflammation and its secreted by many cells including T helper type 2 (Th2) cells (Cocks et al., 1993; Wynn, 2003).

Overall, our data suggest that PCEP and Emulsigen induced cell recruitment which culminated in adjuvant-specific cytokine profiles. These changes may be important in establishing and integrating the immune-competent environment for favorable for antigen

processing, presentation, and subsequent stimulation of antigen-specific immune responses. However, further studies are still needed to evaluate and identify which cell types are essential to induce the appropriate response for any given pathogen. The mechanism of action of vaccine adjuvants must be fully elucidated *in vivo* to make clear how different cell types cooperate in establishing an integrated immunocompetent environment (Mosca et al., 2008b). This information will be critical for the design of new effective vaccines.

Even so, our data reinforces the importance of activating the innate immune responses to establish a robust inflammatory responses which may be beneficial to induction of specific immune response after an immunization. As with Emulsigen, PCEP induced acute but not chronic inflammatory responses at the injection site and the draining lymph nodes when injected into pigs indicating that this adjuvant may be regarded as safe for use in pigs. We speculate that induction of innate immune responses at the site of injection may be an important mechanism through which adjuvant PCEP exert its adjuvant activity in pigs.

Chapter 5. Impact of dose reduction of adjuvant

poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) on inflammatory responses, immunogenicity and safety when co-administered with inactivated swine influenza virus vaccine in pigs

Relationship of this chapter to the dissertation

In chapter 4, we have provided evidence that adjuvant poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) induces cytokine production leading to inflammation at the site of injection. However, too much inflammation can render a vaccine unsafe. If the dose of an adjuvant can be reduced without impacting vaccine efficacy, the results could be improved vaccine safety (less signs of inflammation at the site of injection) and reduced vaccine cost. Because i.d. injection of PCEP induced signs of necrosis (cell death), we investigated whether reduction of the adjuvant dose reduced tissue damage without negatively impacting the antigen-specific immune responses. To address this issue we performed two studies. In the first study, we injected i.d. varying doses of PCEP alone as follows: 500 µg, 100 µg, or 20 µg PCEP into piglets and evaluated the inflammatory responses (granuloma formation, lymphocytic infiltration, necrosis, and suppurative inflammation) at the injection site and the draining lymph nodes over a period of 14 days. When PCEP was injected alone, we observed that only 500 µg consistently induced significant necrosis and suppurative inflammation. However, the medium dose (100 µg) PCEP did induce significant skin granulomas and lymphocyte infiltration, where as the only significant response induced in the skin by the lower dose (20 µg) PCEP was lymphocyte infiltration. In the draining lymph nodes, only 500 ug PCEP significantly higher suppurative inflammation. No necrosis or granuloma was observed in the lymph nodes in all the doses. Thus, the high dose of adjuvant triggered the most significant pathological signs of tissue damage at both sites (skin and draining lymph nodes).

In the second study, we co-injected the varying doses of PCEP 500 µg, 100 µg, 20 µg, or 4 µg with an inactivated swine influenza virus H1N1 antigen and evaluated the four parameters of inflammatory response (granuloma formation, lymphocytic infiltration, necrosis, and suppurative inflammation) at days 20 and 41 after a single injection at each site and also assayed SIV H1N1-specific antibody titers. We observed that the highest dose of the adjuvant PCEP (500 µg) induced significant inflammatory responses in 3 of the 4 parameters assessed at day 20, and by day 41, this high dose of adjuvant had caused significant inflammatory response in all the four parameters assessed. Interestingly, only 500 µg of PCEP induced significant granuloma formation and necrosis (the more severe lesions) at both time points. Of the lower doses, 100 µg and 20 µg of PCEP both induced significant lymphocytic infiltration but their responses were similar. The lowest dose, 4 µg of PCEP did not induce any significant inflammatory response in any of the four parameters assessed. When SIV H1N1-specific antibody titers were assessed in immunized animals, the three higher doses (500 µg, 100 µg and 20 µg) of PCEP induced significant antibody responses when compared to SIV H1N1, confirming that these doses had adjuvant activity. In contrast, the lowest dose (4 µg) of PCEP did not induce significant titers, indicating that this dose did not have adjuvant activity. These results suggest that induction of inflammatory responses at the site of injection is necessary for adjuvant activity. However, not all responses assessed may be required for adjuvant activity as very severe inflammatory responses seen with 500 µg of PCEP was not associated with any additional increase in antibody responses. Thus, the quality of the inflammatory response is important but severe inflammation was not beneficial to antigen-specific immune responses.

In this manuscript my primary role was conception of ideas, design, planning and conducting experiments, analysis and interpretation of the data. I also wrote the initial drafts of the

manuscript, and revised the manuscript based on feedback from other authors. We plan to submit a manuscript of this work for publication.

5.1 Introduction

Because subunit vaccines contain highly purified antigens, they are often poorly immunogenic and require the addition of adjuvants to induce protective immune responses. The ideal adjuvant should maximize vaccine immunogenicity without compromising tolerability or pose undue risk to the recipient of the vaccine. Unfortunately, adjuvant research has lagged behind other vaccine areas such as antigen discovery, with the consequence that only a very limited number of adjuvants based on aluminum salts, monophosphoryl lipid A and oil emulsions are currently approved for human use (Wang & Singh, 2011). Despite their critical role in vaccines, adjuvant safety and toxicity remain poorly understood which is a barrier to rational development of safe and effective vaccines. The inflammatory or danger-signal model of adjuvant action implies that increased vaccine reactogenicity at the site of injection is a price for improved immunogenicity (Petrovsky & Aguilar, 2004a).

Polyphosphazenes are high-molecular weight, water-soluble polymers that promote and enhance long lasting immune responses with a variety of viral and bacterial antigens (Awate et al., 2012; Eng et al., 2010; Magiri et al., 2018b; McNeal et al., 1999; Mutwiri et al., 2008; Mutwiri et al., 2007). PCEP has been shown to promote a stronger mixed Th1/Th2 type of responses relative other polyphosphazene derivatives and therefore PCEP may promote a more broad-spectrum immunity (Mutwiri et al., 2008; Mutwiri et al., 2007). PCEP promotes cytokine and chemokine production as well as regulation of a number of ‘adjuvant core response genes’ at the intramuscular injection site in mice and pigs (Awate et al., 2012; Magiri et al., 2016; Mutwiri

et al., 2008). These responses may contribute to immune cell recruitment to the injection site and transport of antigens to draining lymph nodes (Awate et al., 2014a).

Inflammatory responses induced by tissue necrosis at the site of injection by adjuvants was advanced by (Matzinger, 1994) in her 'danger signal' hypothesis. Danger signals from damaged cells can trigger activation of the immune system through activation of inflammasome leading to release of pro-inflammatory cytokine such as IL-1 β (Kool et al., 2008). Molecules such as uric acid, nucleotides, adenosine triphosphate (ATP), reactive oxygen intermediates, and cytokines are released from cells if there is tissue damage (Krysko et al., 2011). This sterile/semi-sterile inflammation can be induced in response to injection of adjuvants and release of these endogenous molecules can attract inflammatory cells and promote induction of innate immunity (Chen et al., 2007; Kono et al., 2014; Shi et al., 2003). In addition, many adjuvants including PCEP induce release of pro-inflammatory cytokines at the site of injection (Awate et al., 2012; Calabro et al., 2011; Didierlaurent et al., 2009). The effectiveness of PCEP and its antigen dose sparing effects have been demonstrated with various vaccines (Andrianov et al., 2009b; Andrianov et al., 2011a; Mutwiri et al., 2007a). As highlighted by acellular pertussis vaccines, the improved safety of subunit vaccines comes at the price of reduced immunogenicity (Poolman, 2014). There is a need to draw a clear distinction between acceptable range of adjuvant-related side effects and optimal immune response (Gupta & Siber, 1995). It is important to receive sufficient vaccine for the induction of immune response but not to exceed the maximal safe dose as some adjuvants can cause adverse effects at the site of injection such as pyrexia, muscle weakness, arthralgia, myalgia and erythema (Cerpa-Cruz et al., 2013; Petrovsky & Aguilar, 2004a).

There is a general consensus that local inflammation contributes to adjuvant activity. However, details of the relationship between local inflammation and antigen-specific immune

responses have not been investigated. Our previous research established that a high dose of PCEP (500 µg) or a low dose of PCEP (20 µg) co-administered with swine influenza antigen H1N1 antigen triggered equivalent antibody responses (Magiri et al., 2018b). In the current study, we investigated the effect that reduction of adjuvant dose had on the local inflammatory responses and the antigen-specific immune response to inactivated swine influenza vaccine. Further, we investigated whether reduced dose of PCEP reduced pathological tissue damage at the injection site and the draining lymph nodes as a measure of safety of adjuvant PCEP. We hypothesized that induction of inflammatory responses at the site of injection is an important mechanism through which PCEP exert its adjuvant activity in pigs and that reduced adjuvant doses may lead to less histopathological damage without impacting the immune response to swine influenza vaccine.

5.2 Materials and methods

5.2.1 Adjuvant and vaccine preparation

PCEP was synthesized by the Idaho National Laboratory (Idaho Falls, ID, USA) using methods previously described in (A. K. Andrianov et al., 2004; Mutwiri et al., 2007b) and dissolved in endotoxin-free, sterile phosphate-buffered saline (PBS), pH 7.4 (Life Technologies, Carlsbad, CA, USA). Inactivated SIV H1N1 and PCEP were diluted with sterile PBS, pH of 7.4. The highest dose of PCEP (500 µg per dose) was chosen based on the previous experiments in pigs (Dar et al., 2012; Magiri et al., 2016; Magiri et al., 2018b).

5.2.2 Animal experiments

All animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals as indicated by the Canadian Council on Animal Care and was approved

by the Animal Care Committee of the University of Saskatchewan. For all the trials, three to four weeks old Landrace commercial crossbreed pigs were used.

In the first study, we assessed whether the dose of PCEP impacted the inflammatory responses in absence of the antigen. Six groups (n=5 pigs/group) injected i.d. with 4 µg (lowest), 20 µg (low), 100 µg (moderate), 500 µg (high) PCEP or PBS. The injection volume to dissolve the adjuvant was consistently 250 µl per sites. An 8 mm skin punch plug biopsy was collected at 1, 4, 7, and 14 days post injection in 10% formalin for histopathology evaluation to determine whether PCEP induces inflammatory responses in a dose dependent manner.

The purpose of the second trial was to determine the optimal constant dose of antigen to be used in subsequent experiments. Piglets (n=5) were immunized i.d. with either 4×10^4 , 2×10^5 , 1×10^6 SIV H1N1 HAU with 500 µg PCEP with PBS or naive as control. Serum was collected 0, 21, 30 and 35 days post vaccination for assay of antibody response against SIV H1N1. The 4×10^4 Inactivated SIV H1N1 HAU dose was selected for subsequent experiments.

The purpose of the third trial was to further optimize the antigen dose with reduced PCEP co-formulated with the vaccine. Piglets (n=6) were immunized i.d. with 4×10^4 and 8×10^3 inactivated SIV H1N1 HAU with 100 µg PCEP, or PBS or naive animals as controls. Serum was collected 0, 21, 30 and 35 days post vaccination for assay of antibody response against SIV H1N1. The 4×10^4 Inactivated SIV H1N1 HAU dose was selected for subsequent experiments.

In the fourth study, to determine adjuvant dose response to swine influenza virus vaccine and the injection responses with constant doses of antigen, piglets were divided into 6 test groups (n = 6 in each group). Pigs were immunized i.d. with constant 4×10^4 inactivated SIV H1N1 HAU alone or with 4, 20, 100, 500 µg PCEP or PBS. Vaccination was performed on day 0 and a booster vaccination was given on day 21. Serum was collected 0, 21, 28 and 35 days post vaccination for assay of antibody response against SIV H1N1. The piglets were anaesthetised

with 5% isoflurane and anaesthesia maintained using 2% isofluorane then an endotracheal tube was inserted to about 2/3 of the trachea length just above the bifurcation and challenged with 4 mL of 8×10^5 PFU H3N2 in MEM media/pig. Animals were euthanized 5 days post-infection (41 days post primary immunization) using Euthanyl (25 mg sodium pentobarbital) administered intravenously (I.V.). The i.d. injection site at initial and booster vaccination site were collected in formalin for histopathology evaluation to assess whether reduction in the dose of the adjuvants lead to the reduction in tissue damage and whether the tissue damage was resolved 41 days after vaccination.

5.2.3 Assessment of intradermal site reaction

Skin punch biopsy tissues were prepared for histopathological evaluations as follows: Tissues were fixed with 10% formalin for 2 weeks then incubated for 24 hour in 70% ethanol, 100 % ethanol and finally 100% xylol to clear the tissues. Tissues were then embedded in paraffin block for easier sectioning in the microtome and sliced to 3 to 5 μ m thickness. Slides were stained using standard Haematoxylin-eosin method following the standard operating procedures. We evaluated changes in histopathology including granuloma formation (microscopic aggregation of macrophages transformed into epithelium like cells surrounded by a collar of mononuclear leukocytes, principally lymphocytes, and plasma cells), lymphocytes infiltration (infiltration of tissue by T and B lymphocytes), epithelial necrosis (death of cells and living tissues), and suppurative inflammation (forms as a result of the action of polymorphonuclear_leucocytes e.g. neutrophils). The histopathological changes of the skin included granuloma formation, lymphocytes infiltration, epithelial necrosis, and suppurative inflammation whereas histopathological changes in the draining lymph nodes included granuloma formation and suppurative inflammation. Scoring was performed by a pathologist

blinded to the treatment groups. Scoring was as follows: A) No pathological changes = 0, B) Patchy pathological changes = 1, C) Moderate pathological changes = 2, D) Severe pathological changes = 3 as described by (Magiri et al., 2018b; Mikalsen et al., 2012)

5.2.4 Detection of Swine Influenza Virus H1N1 antibodies in porcine serum by enzyme-linked immunosorbent assay (ELISA).

Purified SIV H1N1 was inactivated by mixing one part virus with one part 5% N-lauroyl sarcosine sodium salt (Sigma-Aldrich Canada Co. Oakville, Ontario) and 8 parts PBSA + 0.1% sodium azide for 30 min at room temperature. Virus was diluted to 0.5 µg/mL in 0.5 M bicarbonate buffer then applied to Immulon 2 96U plates (Thermo Lab systems #3655) at 100 µL per well and the plates were incubated at 4°C overnight. Coated plates were washed extensively with tris-buffered saline plus 0.05% Tween-20 (TBST; Sigma). Serum samples and control were diluted in TBST, transferred into coated plates, and diluted serially 4-fold serially. Antigen-specific total IgG were detected with alkaline phosphatase conjugated KPL goat anti-swine IgG (H+L) (Invitrogen) and developed using PNPP substrate. Optical density was read at λ405 nm and the final titers were calculated using an excel spreadsheet.

5.2.5 Statistical analysis

Statistical analyses were assessed using Graph-Pad Prism 7 software (GraphPad Software, San Diego, CA, USA). Analysis of site reaction scores values between different treatment groups were compared using non-parametric Kruskal-Wallis ANOVA test where Dunn's multiple comparisons test was used post-hoc to identify statistically significant differences between different adjuvant test groups. ELISA antibody and serum antibody titers were ranked using repeated measures, before comparison of treatment groups using the above mentioned statistical

analysis were utilized. $P < 0.05$ values was considered to be statistically significant.

5.3 Results

5.3.1 Dose dependent inflammatory responses induced by adjuvant PCEP in absence of the antigen.

We evaluated by histopathology how the dose of adjuvant impacted inflammatory responses including granuloma formation (microscopic aggregation of macrophages transformed into epithelium-like cells surrounded by a collar of mononuclear leukocytes, principally lymphocytes, and plasma cells), lymphocytes infiltration (infiltration of tissue by T and B lymphocytes), epithelial necrosis (death of cells and living tissues), and suppurative inflammation (which forms as a result of the action of recruited polymorphonuclear leucocytes e.g. neutrophils). High (500 μg) and medium (100 μg), and low (20 μg) doses of PCEP (or PBS) were injected i.d. into pigs to assess inflammatory responses at the injection site and the draining lymph nodes on 1, 4, 7 and 14 day post injection. Body temperature was recorded and injection sites were examined for signs of lesions. In all the animals, no macroscopic lesions at the sites of injection were observed and body temperatures remained normal throughout the study period suggesting that the adjuvants PCEP did not induce a systemic immune response (data not shown). We observed that only 100 μg PCEP induced significant granuloma formation 14 days post-administration ($p < 0.01$) compared to PBS. All the other groups were not significantly different from each other at all the time points (Fig 13A). With regard to necrosis, the high dose of PCEP (500 μg) induced significant necrosis at 1, 4, and 7 days ($p < 0.01$) post-administration relative to PBS controls (Fig 13B). This high dose of PCEP (500 μg) was significantly higher than low dose (20 μg) at day 1 post injection (Fig 13B; $p < 0.01$) but all other groups were not significantly

different from each other at all the time points. With regard to suppurative inflammation, the high dose of PCEP (500 µg) induced significant suppurative inflammation from day 1, 7 and 14 days post injection ($p<0.01$, $p<0.01$ and $p<0.01$ respectively) relative to the PBS controls (Fig 13C) while the medium dose of PCEP (100 µg) induced significant suppurative inflammation at 4 days ($p<0.05$) post administration relative to the PBS controls (Fig 13C). The low dose (20 µg) of PCEP induced significant suppurative inflammation 4 days ($p<0.05$) post administration relative to the PBS controls (Fig 13C). This high dose of PCEP (500 µg) was significantly higher suppurative inflammation than low dose (20 µg) at day 1 post injection (Fig 13C; $p<0.01$) but all other treatment groups were not significantly different from each other at all the time points. With regard to lymphocytic infiltration, the Medium dose (100 µg) of PCEP induced significant infiltration at 1, 7 and 14 days post-administration (Fig 13D; $p<0.05$) and low dose (20 µg) of PCEP induced significant infiltration at 4, and 7 days post-administration relative to PBS controls (Fig 13D; $p<0.01$ $p<0.05$). The high dose PCEP (500 µg) induced significant lymphocytic infiltration at 14 days ($p<0.01$), post-administration relative to saline controls but all the treatment groups were not significantly different from each other at all the time points (Fig 13D).

At the draining lymph nodes, on granuloma formation we observed that all treatment groups were not significantly different from each other at all the time points (data not shown). The high dose of PCEP (500 µg) had significantly higher suppurative inflammation than the low dose (20 µg) at day 1 post injection but all other treatment groups were not significantly different from each other at all the time points (data not shown).

Generally, the higher adjuvant dose (500 µg of PCEP) triggered the most significant pathological signs of tissue damage at both the injection sites in the skin and the draining lymph nodes.

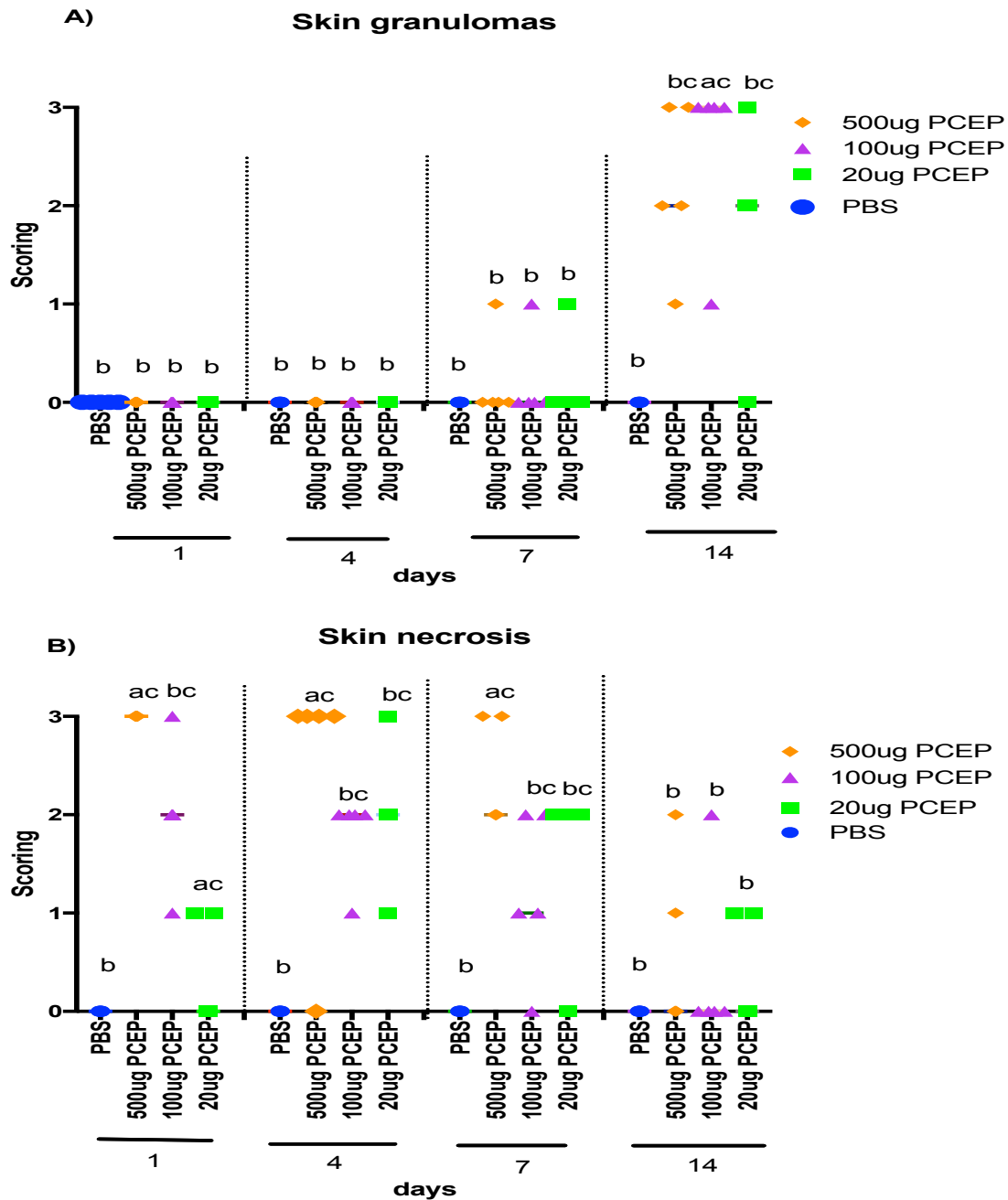


Figure 5.1 A and B The histopathological changes of the skin injection site in response to PCEP at the site of intradermal administration in pigs. An 8 mm skin punch plug biopsy was collected from one of the sites at 1, 3, 7, and 14 days post injection. Results shown are the SEM of five replicates at each time point compared to PBS injected skin tissue. The histopathological changes of the skin included were granuloma formation, lymphocytes infiltration, epithelial necrosis, and suppurative inflammation. The groups with different letters are significantly different from each other at that time point ($P < 0.05$).

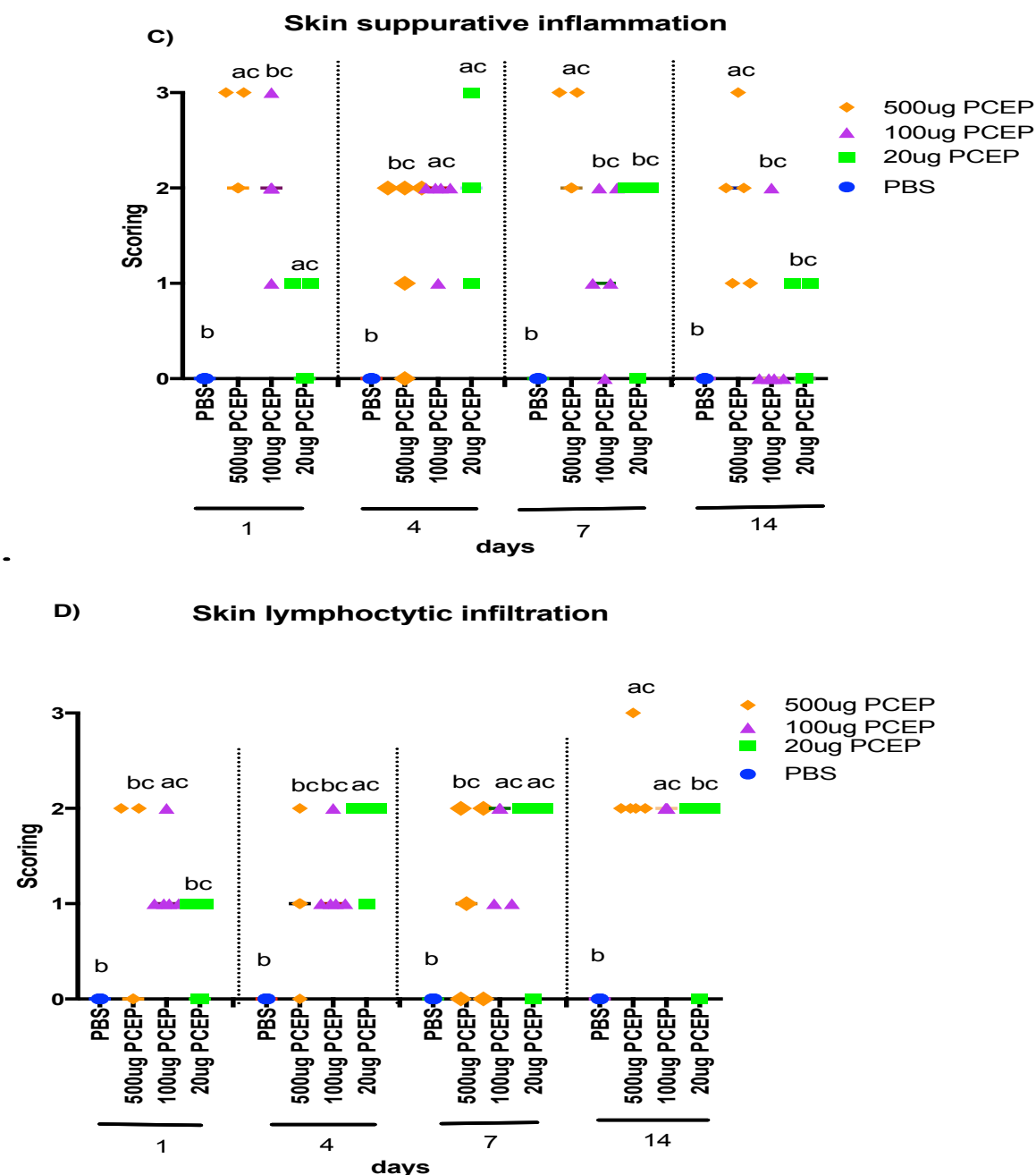


Figure 5.1 C and D The histopathological changes of the skin injection site in response to PCEP at the site of intradermal administration in pigs. An 8 mm skin punch plug biopsy was collected from one of the sites at 1, 3, 7, and 14 days post injection. Results shown are the SEM of five replicates at each time point compared to PBS injected skin tissue. The histopathological changes of the skin included were granuloma formation, lymphocytes infiltration, epithelial necrosis, and suppurative inflammation. The groups with different letters are significantly different from each other at that time point ($P < 0.05$).

5.3.2 Selection of the dose of antigen.

To determine the dose of antigen for subsequent experiments, piglets were injected with i.d. with a vaccine consisting of either 4×10^4 , 2×10^5 , 1×10^6 SIV H1N1 HAU with 500 μ g PCEP or with PBS and pigs without injection (i.e. naïve pigs) were used as controls. A booster immunization was administered 21 days after the primary immunization and sera were collected 0, 21, 30 and 35 days post vaccination to assess antigen-specific antibody response. Piglets immunized with 4×10^4 , 2×10^5 , 1×10^6 SIV H1N1 HAU formulated with 500 μ g PCEP showed significantly higher ($P < 0.05$, $p < 0.01$ and $p < 0.01$, respectively) serum antibody titres (Fig 14).

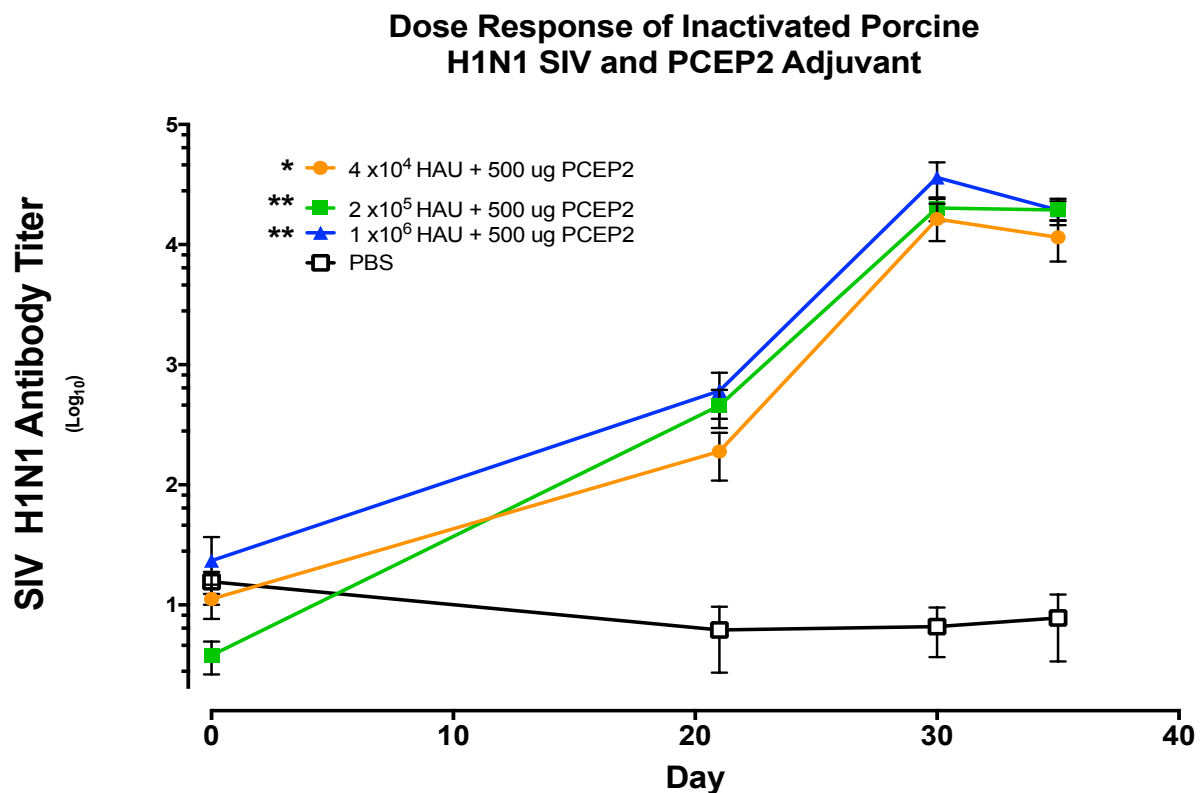


Figure 5.2 To determine the constant dose of antigen to be used in subsequent experiments. Piglets (n=5) were immunized i.d. with either 4×10^4 , 2×10^5 , 1×10^6 SIV H1N1 with 500 μ g PCEP with PBS or naïve as control. ELISA antibody and serum antibody titers were ranked using repeated measures. The area under the curve (AUC) was calculated using a non-parametric non-parametric Kruskal-Wallis ANOVA test where Dunn's multiple comparisons test was used post-hoc to identify statistically significant differences between different adjuvant test groups. $P < 0.05$ values was considered to be statistically significant.

To optimize the antigen dose, piglets were immunized with 4×10^4 and 8×10^3 inactivated plus 100 μ g PCEP. Both 4×10^4 and 8×10^3 vaccine doses showed significantly higher ($P < 0.01$, $P < 0.05$) serum antibody titres relative to the negative controls animals (Fig 15) so the 4×10^4 inactivated SIV H1N1 HAU dose was selected for subsequent experiments.

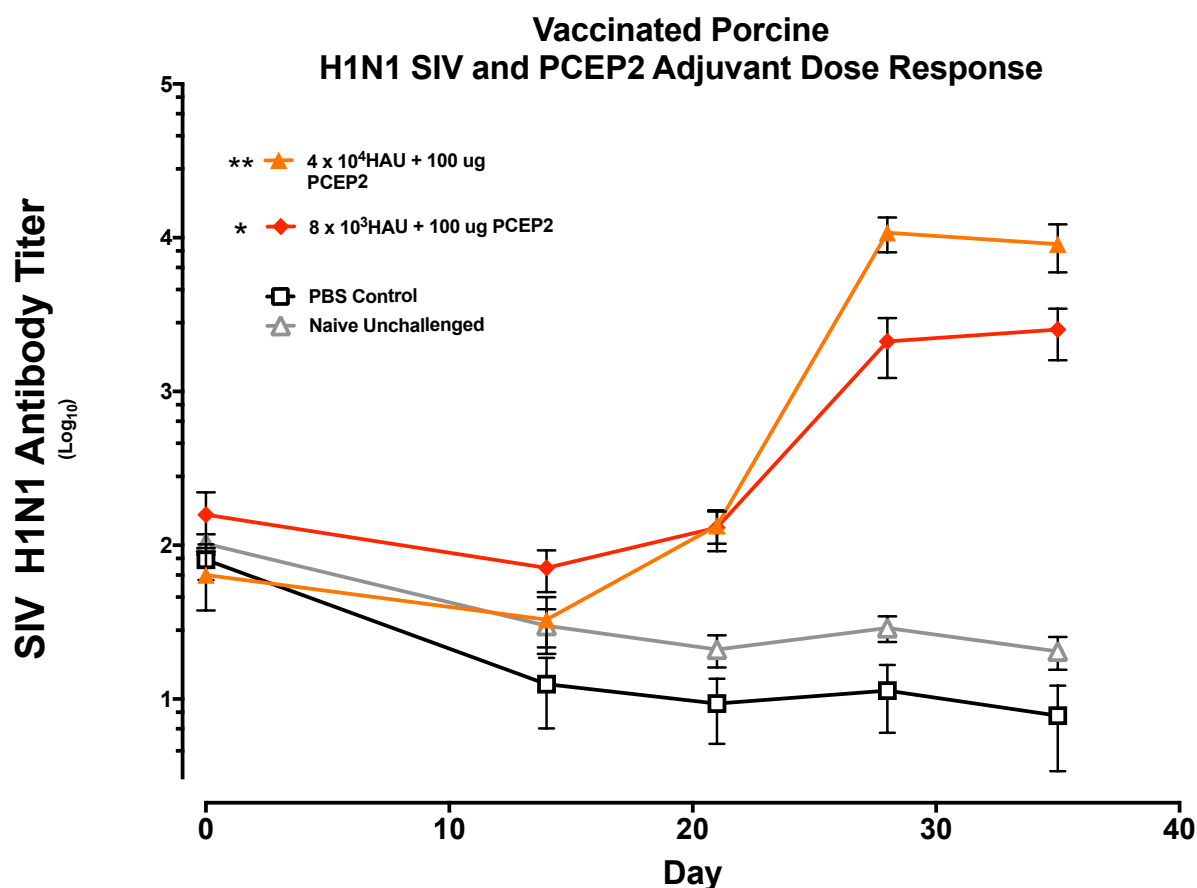


Figure 5.3 Antigen dose titration of inactivated SIV H1N1 for use in subsequent experiments. To optimize the dose of the antigen piglets were immunized with 4×10^4 and 8×10^3 inactivated plus 100 μ g PCEP i.d. Serum was collected 0, 21, 30 and 35 days post vaccination for assay of antibody response against SIV H1N1. ELISA antibody and serum antibody titers were ranked using repeated measures. The area under the curve (AUC) was calculated using a non-parametric non-parametric Kruskal-Wallis ANOVA test where Dunn's multiple comparisons test was used post-hoc to identify statistically significant differences between different adjuvant test groups. $P < 0.05$ values was considered to be statistically significant

5.3.3 Reduced doses of PCEP induces immune responses against inactivated swine influenza virus H1N1.

Pigs were injected i.d. with PBS or 4×10^4 Inactivated SIV H1N1 HAU alone or plus 4, 20, 100, 500 μg PCEP at day 0 then boosted 21 days later. Serum samples were collected over a period of 35 days and antigen-specific serum antibody titres were assayed by ELISA. Negligible antibody titres were detected in piglets immunized with inactivated SIV alone or in the presence of adjuvants by day 20 prior to the second immunization suggesting that the primary immunization was not sufficient (Fig 16). After 35 days, pigs immunized with 4×10^4 inactivated SIV H1N1 HAU plus 20, 100, 500 μg of PCEP showed significantly higher ($P < 0.01$ $P < 0.001$, $P < 0.01$, respectively) serum antibody titres relative to the negative controls animals. Interestingly, reduction in the dose of adjuvant did not alter the magnitude of immune response as 20 μg , 100 μg and 500 μg of PCEP triggered similar antigen-specific immune responses to SIV H1N1 antigen ($P < 0.01$, $P < 0.01$, respectively). These findings indicate that a reduced amount of PCEP was still successful in inducing significant antibody responses without significant pathological reaction at the site of injection.

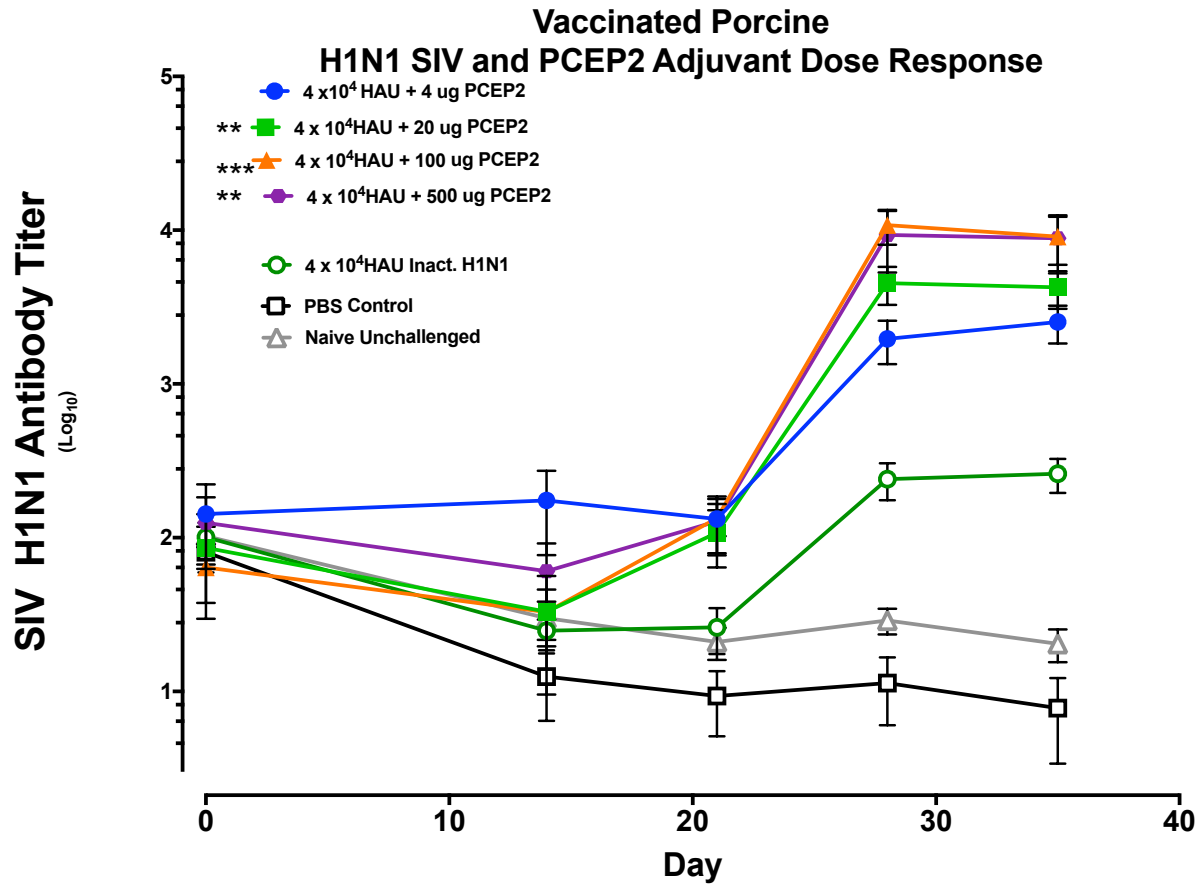


Figure 5.4 Reduced doses of PCEP induce immune responses against inactivated swine influenza virus H1N1. Pigs were injected i.d. with PBS or 4×10^4 Inactivated SIV H1N1 HAU alone or plus 4, 20, 100, 500 μ g PCEP at day 0 then boosted 21 days later. Serum samples were collected over a period of 35 days and antigen-specific serum antibody titres were assayed by ELISA. ELISA antibody and serum antibody titres were ranked using repeated measures. The area under the curve (AUC) was calculated using a non-parametric non-parametric Kruskal-Wallis ANOVA test where Dunn's multiple comparisons test was used post-hoc to identify statistically significant differences between different adjuvant test groups. $P < 0.05$ values was considered to be statistically significant

5.3.4 Pathological reaction at the site of injection following vaccination with Inactivated SIV H1N1 HAU with varying doses of adjuvant the primary and booster immunization sites.

Pigs were injected i.d. with a constant dose antigen 4×10^4 inactivated SIV H1N1 HAU formulated with either 4, 20, 100, 500 μg PCEP of adjuvant per injection in 250 μL sterile PBS. The sites of injection were collected in formalin for histopathology evaluation. Animals were immunized and euthanized such that one immunization site was collected 41 days post injection and another immunization site was collected 20 days post-injection (See Fig 17).

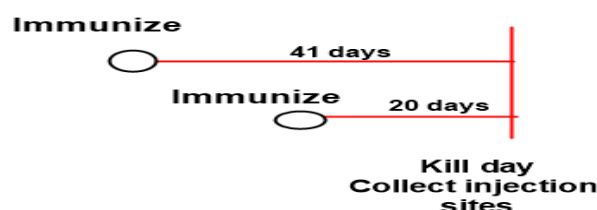


Figure 5.5 Immunization protocol

We observed that, on day 20, 500 μg PCEP induced significantly higher granuloma formation than all other groups (Fig. 18A). All other groups were not significantly different from each other at day 20. Similar observations were made on 41, where 500 μg of PCEP induced significantly higher granuloma formation than all other groups. All other groups were not significantly different from each other (Fig. 18A). Thus only 500 μg of PCEP induced significant granuloma formation time points.

When necrosis was assessed, only 500 μg PCEP induced significantly higher necrosis than all other groups on day 20 (Fig. 18B). All other groups are not significantly different from each other at day 20. Similar findings were observed at day 41 where 500 μg PCEP induced

significantly higher necrosis formation than all other groups. All other groups are not significantly different from each other (Fig. 18B)

On day 20, only 100 µg of PCEP induced significantly higher suppurative inflammation than 4 µg PCEP, SIV and PBS while all other groups were similar (Fig. 18C). On day 41, 500 µg PCEP induced significantly higher suppurative inflammation than all other groups. All other groups were not significant different from each other. Interestingly, on day 20, only 100 µg PCEP induced a significant response, which subsided by day 41. In contrast, this response was delayed till day 41 in animals injected with 500 µg PCEP (Fig. 18C)

On lymphocytic infiltration on day 20, 500 µg, 100 µg and 20 µg of PCEP induced significantly higher lymphocyte infiltration than 4 µg PCEP, SIV and PBS. 4 µg of PCEP did not induce significant lymphocyte infiltration compared to SIV and PBS. Additionally SIV did not induce significant lymphocyte infiltration compared to PBS. Therefore 500 µg, 100 µg and 20 µg PCEP induced similar lymphocytic infiltration responses that are significantly stronger than 4 µg PCEP, SIV and PBS. The latter 3 groups are similar with no significant lymphocytic infiltration (Fig. 18D). Interestingly, on day 41, only 500 µg PCEP induced significantly higher lymphocyte infiltration than all other groups. All other groups were not significantly different from each other hence lymphocytic infiltration had subsided by day 41 in all groups except 500 µg PCEP (Fig. 18D)

In summary, the highest dose of the adjuvant PCEP (500 µg) induced significant inflammatory responses in 3 of the 4 parameters assessed at day 20, and day 41, this high dose of adjuvant caused significant tissue response in all four parameters assessed. Interestingly, only 500 µg of PCEP induced significant granuloma formation and necrosis (the more severe lesions) at both time points. Of the lower doses, 100 µg and 20 µg of PCEP both induced significant lymphocytic infiltration but their responses were similar.

The lowest dose, 4 µg of PCEP did not induce any significant inflammatory response in any of the four parameters assessed. Decreased granulomatous inflammation, necrosis, lymphocyte infiltration and suppurative inflammation was observed with decreasing concentration of PCEP in the i.d. vaccine indicating that reduction of the dose of adjuvant in vaccine can lead to reduction in tissue damage without reduction in the magnitude of the antigen-specific immune response.

When SIV H1N1-specific antibody titers were assessed in immunized animals, only 500, 100 and 20 µg of PCEP which had significant inflammatory responses had also developed significant antibody responses when compared to SIV H1N1, confirming that these doses had adjuvant activity (Fig. 16). However, 4 µg of PCEP did not induce significant inflammatory responses and SIV H1N1-specific antibody titers, indicating that this dose did not have adjuvant activity.

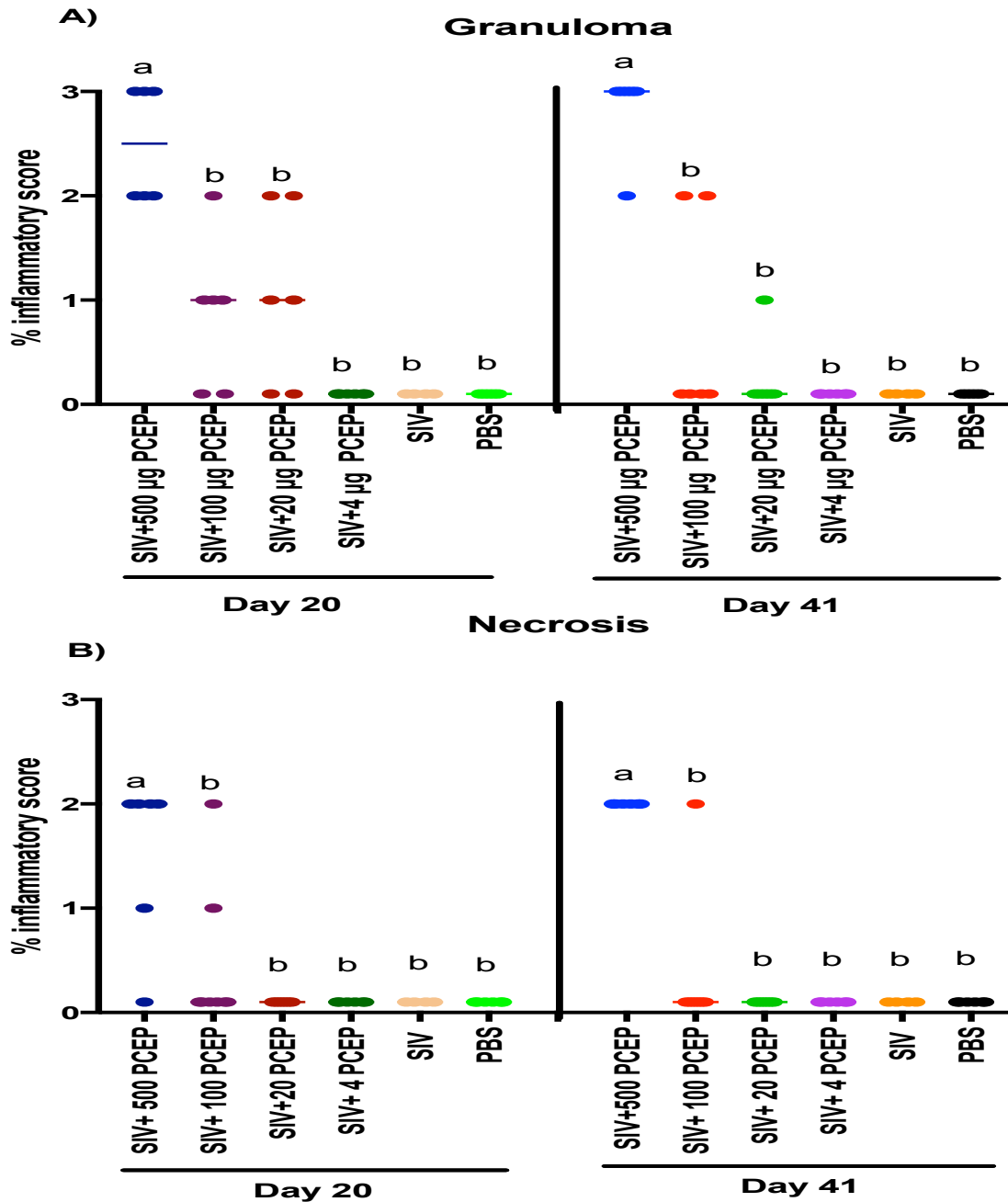


Figure 5.6 A and B Pathological reaction at the site of injection following vaccination with Inactivated SIV H1N1 with varying doses of adjuvant the primary and booster immunization sites. Pigs were injected i.d. with a constant dose antigen 4×10^4 inactivated SIV H1N1 HAU and formulated with 20, 100, 500 µg PCEP of adjuvant per injection. The sites of injection were collected in formalin for histopathology evaluation. The primary site of injection was therefore collected 41 days later and the booster injection site was collected only 20 days post-injection. The groups with different letters are significantly different from each other at that time point ($P < 0.05$).

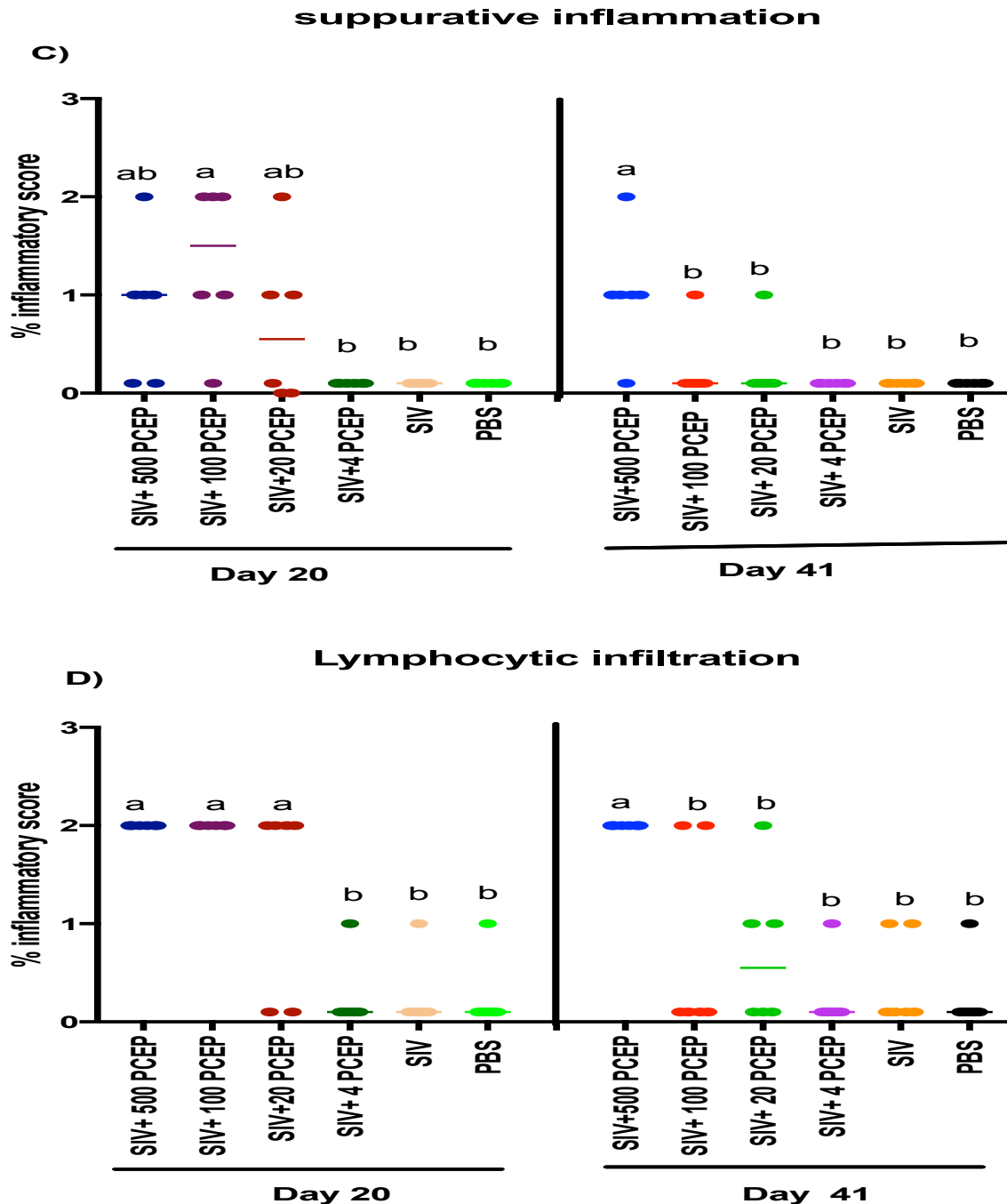


Figure 5.6 C and D Pathological reaction at the site of injection following vaccination with Inactivated SIV H1N1 with varying doses of adjuvant the primary and booster immunization sites. Pigs were injected i.d. with a constant dose antigen 4×10^4 inactivated SIV H1N1 HAU and formulated with 20, 100, 500 μ g PCEP of adjuvant per injection. The sites of injection were collected in formalin for histopathology evaluation. The primary site of injection was therefore collected 41 days later and the booster injection site was collected only 20 days post-injection. The groups with different letters are significantly different from each other at that time point ($P < 0.05$).

5.4 Discussion

Adjuvants are crucial components in vaccines that broadens the immune responses, particularly for the poorly immunogenic subunit protein antigens (Reed et al., 2013b). Subject to the adjuvant's nature, immune responses can be enhanced and/or skewed towards a particular cellular and/or humoral responses and promote infiltration of specific cell populations (Guy, 2007). Improving the benefits (efficacy) of immunization while decreasing their potential risks (adverse reactions) is essential in the development of all new vaccines and is a key factor driving new technologies and sophisticated vaccine design. By counteracting the poor immunogenicity of purified antigen, the addition of adjuvants to vaccines may lead to an increase in pain, redness, and swelling at the site of injection and sometimes general symptoms such as fatigue, malaise, myalgia and fever (Garçon et al., 2011; Garçon et al., 2011). Vaccine reactogenicity is regarded as a dose-dependent phenomena reflecting local tissue damage and systemic inflammation induced by activation of innate immune receptors (Petrovsky & Aguilar, 2004a). The reactogenicity profile of any adjuvanted vaccine is specific to the antigen and the target population studied (Kosalaraksa et al., 2014; Mark & Granström, 1994; Waddington et al., 2010). Should an adjuvant induce excessive reactogenicity in a vaccine that cannot be resolved by reducing the dose of the reactogenic component, then the adjuvanted vaccine could be regarded as potentially unsafe.

Local vaccine side effects may reflect direct chemical irritation due to non-physiological pH, osmolarity, salt concentrations or direct cell toxicity. Such local irritant effects are typically associated with immediate severe injection site pain followed by an inflammatory response triggered by the tissue damage. Examples of adjuvants that induce local reactogenicity include saponins (e.g. Quil A, QS21, ISCOMS, ISCOMATRIX) and oil emulsions (e.g. CFA, IFA, Montanide, MF59, AS03)(Aguilar & Rodriguez, 2007; Aucouturier et al., 2001; Petrovsky &

Aguilar, 2004a; Zhu et al., 2013). In the present study, we investigated whether reduction of PCEP dose had any effect on reactogenicity to the vaccine at the site of injection. Further, we investigated whether reduced dose of PCEP could lead to reduced evidence of pathological tissue damage at the injection site and the draining lymph nodes as a measure of safety. We show that when adjuvant alone was injected i.d., inflammatory cells (dominated by neutrophils at first, followed by lymphocytes and later macrophages) were recruited to the injection site between 4 and 14 days after injection in all the PCEP doses. Interestingly, only 500 µg PCEP induced significant necrosis that persisted past 7 days suggesting that reduction of adjuvant dose led to reduced tissue damage. When PCEP was co-administered with inactivated swine influenza virus antigen, the vaccine induced systemic antibody production even at low doses. Other studies have shown induction of inflammation by aluminium-containing adjuvants is important for the recruitment of antigen-presenting cells and the release of cytokines and other mediators that induce maturation and activation of dendritic cells has been reported (Greaves, 2011). Overall, the results of other studies that have compared vaccines with and without adjuvant have shown a consistent trend toward increased reactogenicity, mainly at the injection site with adjuvanted formulations (Garçon et al., 2011; Garçon et al., 2011). We suggest that adjuvant PCEP appears to be responsible for the inflammatory responses at the site of injection that are critical in initiation of the immune responses even with reduced doses. Indeed, our recent studies have indicated that PCEP plays a role in the recruitment of neutrophils, natural killer cells, macrophages, eosinophils and immature dendritic cells to the site of injection and draining lymph nodes (Awate et al., 2012; Awate et al., 2014; Magiri et al., 2016) which in turn promotes the adaptive immune response to a vaccine (Magiri et al., 2018a).

When tissue is damaged, the period of time in which residual inflammation and the associated cellular debris is restored to normal structure is called 'recovery' (Perry et al., 2013;

Thuilliez et al., 2009). In the second study, intradermal injection sites were evaluated after injection with varying doses of PCEP as part of a SIV H1N1 vaccine and the four parameters of inflammatory response (granuloma formation, lymphocytic infiltration, necrosis, and suppurative inflammation) assessed at days 20 and 41 after a single injection at each site. The highest dose of the adjuvant PCEP (500 µg) induced significant inflammatory responses in 3 of the 4 parameters assessed at day 20, and by day 41, this high dose of adjuvant caused significant tissue response in all four parameters assessed. Interestingly, only 500 µg of PCEP induced significant granuloma formation and necrosis (the more severe lesions) at both time points. Of the lower doses, 100 µg and 20 µg of PCEP both induced significant lymphocytic infiltration compared to PBS but responses of the two adjuvants groups were similar. The lowest dose of PCEP (20 µg) did not induce any significant inflammatory response in any of the four parameters assessed.

When SIV H1N1-specific antibody titers were assessed in immunized animals, only 500, 100 and 20 µg of PCEP induced significant antibody responses when compared to SIV H1N1, confirming that these doses had adjuvant activity. However, the 4 µg of PCEP did not induce significant titers, indicating that this dose did not have adjuvant activity. Similar to our finding, aluminum-adjuvanted vaccines induced inflammatory nodules at the injection site, which expanded into the intramuscular space without any muscle degeneration or necrosis, whereas non-adjuvanted vaccines did not. These nodules consisted of polymorphonuclear neutrophils with some eosinophils present in the nodes within the initial 48 h, followed by the presence of monocytes/macrophages 1 month post injection (Kashiwagi et al., 2014).

We report that reduction of the dose of adjuvant PCEP led to reduction in tissue reaction in a dose-dependent fashion but did not compromise the immune response to swine influenza virus vaccine (or antigen). Additionally, signs of decreased granulomatous inflammation, necrosis, lymphocyte infiltration and suppurative inflammation were observed with decreasing

concentration of PCEP in the vaccine without negatively impacting the magnitude of the immune response. Recently we also observed that 500 µg PCEP adjuvanted inactivated SIV H1N1 vaccine immunized i.d. triggered some evidence of protective immunity (i.e. neutralizing antibody production and reduced viral load) against homologous challenge even with reduced adjuvant of PCEP by 25 folds in the formulation (Magiri et al., 2018b).

In this study, we show that PCEP concentration of 20 µg (5 folds decrease in dose relative to the highest dose) induced immune responses similar to those seen with 500 µg concentration in swine influenza vaccine, which is consistent with other studies showing that the typical (2.3%) oil dose of the MF59 adjuvant could be reduced by half (but not 4-fold) without compromising immunogenicity and still meet the European criteria for pandemic vaccine licensure (Keitel et al., 2010).

Others showed that reduction of emulsion dose from 2% to 1% or 0.5% (vol/vol) squalene in GLA-SE did not compromise immunogenicity in malaria vaccine (Fox et al., 2012) but they did not report on the effect on tissue reactogenicity. Interestingly, they reported that reduction of the emulsion dose in GLA-SE from 2% to 1% or 0.5% led to higher IgG2a antibody and IFN-γ production hence elicit a strong Th1 type of immune response in mice (Fox et al., 2012). In contrast to our findings with PCEP, a study employing seasonal influenza virus antigen and MF59 at doses of 0.125, 0.25, 0.5, and the full dose in 6- to 36-month-old children found no difference in reactogenicity between the different adjuvant doses (Della Cioppa et al., 2011). In contrast, two different doses of AS03 [AS03A, containing the full dose of 2.5% squalene and 2.5% tocopherol, versus AS03B, containing a half dose (1.25% squalene and 1.25% tocopherol)] when evaluated in another influenza vaccine clinical trial reported that initial immune responses elicited by the two different adjuvant doses were equivalent but the durability of the response measured at 182 days post vaccination was better with the higher adjuvant dose (Langley et al.,

2010).

In conclusion, when the PCEP dose was reduced, we generally observed less histopathological damage without impacting the immune response to an influenza vaccine. These results suggest that induction of inflammatory responses at the site of injection is necessary for adjuvant activity. However, not all responses assessed may be required for adjuvant activity as induction of a very severe inflammatory response was not associated with any additional increase in antibody responses. Thus, the quality of the inflammatory response is important but severe inflammation was not beneficial to antigen-specific immune responses.

CHAPTER 6. GENERAL DISCUSSIONS AND CONCLUSIONS

Vaccination continues to be a very important public health tool in the control of infectious diseases in the world (Andre et al., 2008). Vaccines are estimated to prevent approximately 2.5 million deaths and many more illnesses each year. Vaccines mimic natural infection in the body leading to activation of the immune system so that subsequent encounter with similar antigens will trigger activation of the memory immune responses (Pasquale et al., 2015). PAMPs are constitutively present in infectious agents and can act as vaccine adjuvants that activate PRRs on local innate immune cells such as APCs at the injection site. PRR activation on APCs leads to initiation of a series of signaling events and eventually induction of protective adaptive immune responses to the vaccine antigens (Pashine et al., 2005). Subunit vaccines contain highly purified antigens which themselves are poorly immunogenic (presumably because they lack PAMPs) and require addition of adjuvants to induce protective immune responses (Ulmer et al., 2006). Despite their critical role in vaccines and their use in billions of doses of vaccines for many decades, the MOA of many adjuvants remain poorly understood. The lack of detailed information on how adjuvants work is a barrier to their rational use in vaccines especially in the development of safe and effective vaccines.

Immunoadjuvants enhance and modulate antigen specific immune responses and serve as key components in the development of modern vaccines. This term may generally include either specific immunostimulating molecules, which directly activate innate immune receptors, or delivery systems, which consist of non-immune stimulating components that function as adjuvants by promoting more effective antigen presentation to lymphocytes. Immunostimulatory adjuvants enhance the quality and magnitude of the adaptive immune responses by activating innate immune responses induced by antigens through increased inflammatory responses at the injection site, which are localized, short lived, and without systemic responses (Bachmann &

Jennings, 2010). Secondly, by activating and inducing maturation of APCs, which then traffic to the draining lymph nodes where they direct the type, magnitude and quality of the adaptive immune responses (Dinarello, 2009).

Due to increasing understanding of pathogen interaction with the immune system, the role of adjuvants in modern vaccines is increasingly being understood. Polyphosphazenes are high-molecular weight, water-soluble polymers that have been shown to promote and enhance long lasting immune responses with a variety of viral and bacterial antigens in several animal models (Eng et al., 2010; McNeal et al., 1999; Mutwiri et al., 2008). PCEP promote mixed Th1/Th2 type of immune responses hence leading to broad-spectrum immunity (Mutwiri et al., 2007). Research in our laboratory revealed that the adjuvant PCEP enhances immune responses in various animal species including mice, pigs, sheep and cattle (Eng et al., 2010; Mutwiri et al., 2008).

Recent advances in murine immunobiology indicate that most adjuvants including PCEP act by stimulating the innate immune responses leading to induction of cytokine and chemokine production at the site of injection (Mutwiri et al., 2008), recruitment of immune cells and transport of antigen to the draining lymph node in mice (Awate et al., 2014), increased antigen uptake by DCs and activation and maturation of APCs. Recent reports suggest that PCEP injected either via the i.m. or i.d. routes induced expression of a set of common genes referred to as "adjuvant core response genes" at the site of injection in mice (Awate et al., 2012) and pigs (Magiri et al., 2016). *In vitro*, PCEP stimulates significant production of the innate cytokines IL-12 and IFN- γ suggesting that activation of innate immunity may be important in mediating its adjuvant activity (Mutwiri et al., 2008).

Understanding the mechanisms by which adjuvants mediate their effects will provide critical information on how innate immunity influences the development of adaptive immunity. Despite being a critical vaccine component, the mechanisms by which adjuvants work are not

fully understood and this is especially true when they are used in large animals. This lack of understanding limits our ability to design effective vaccines. As part of this thesis, we administered PCEP or PBS via an i.d. injection into pigs and assessed the impact on the expression of reported ‘adjuvant response genes’ over time and compared the responses to a previously reported study with mice. Studies with mice and pigs revealed species-specific differences in PZ-induced stimulation of innate immune responses (Awate et al., 2012; Magiri et al., 2016). Intramuscular injection of PCEP induced time-dependent changes in the gene expression of many ‘adjuvant core response genes’ (Mosca et al., 2008a) such as chemokine genes CCL-2, CCL-4, CCL-5, CCL-12 and CXCL-10 in mice (Sunita Awate et al., 2012) and CCL-2 and CXCL-10 (but not CCL- 5) in pigs (Magiri et al., 2016). Major transcription factor NF- κ B gene and the inflammatory cytokine TNF- α genes were up-regulated in response to PCEP in mice (Awate et al., 2012) but not in pigs (Magiri et al., 2016). At the protein level, PCEP promoted significant local production of Th1-type proinflammatory cytokines (IL- 1 β , IL-6, IL-18 IFN- γ and TNF- α) and Th2-type cytokines (IL-4 and monocyte chemoattractants CCL-2 and CXCL-10) at the site of injection in mice but not systemically (Awate et al., 2012). However, in pigs, PCEP induced IL-6 gene expression but not IL-10, IL-17 or IFN- α (Magiri et al., 2016). PCEP injection in mice increased the expression of TLR4 and TLR9 at the site of injection (Awate et al., 2012) whereas PCEP did not induce significant expression of any of the TLR genes in pigs suggesting differences in activation of immune responses in different animal species (Magiri et al., 2016). These results suggest that PCEP may modulate antigen-specific immune responses by activating early innate immune responses and promoting a strong immunostimulatory environment at the site of injection. Our studies provide evidence that the effect that adjuvants have on innate immune responses can differ remarkably between species

We previously reported that in mice, injection of PCEP increased cell recruitment and cytokine production at the site of injection with PCEP adjuvant. In this study presented in this thesis, we evaluated whether PCEP induced similar innate immune responses in pigs. Piglets were injected with either PCEP or Emulsigen i.d. and the local cellular infiltration and cytokine production were evaluated at the site of injection and the draining lymph nodes. PCEP induced infiltration of macrophages, T and B cells, leucocytes and necrotic debris at the site of injection as well as PCEP-induced leucocyte infiltration in the draining lymph nodes. Emulsigen induced diffuse infiltration of leucocytes, macrophages, and lymphocytes at the site of injection as well as at the draining lymph nodes. PCEP induced significant production of interleukin IL-1 β , and IL-13 at the site of injection and IL-1 β , and IL-6 at the draining lymph nodes. Emulsigen promoted production of IL-1 β , IL-6, and IL-12 at the site of injection but not in the draining lymph nodes.

These results from pigs are consistent with results in mice from other authors who have determined that adjuvants induce a local microscopic inflammatory reaction at an injection site (Calabro et al., 2011; Schijns, 2000) without gross macroscopic ulcerations (das Does Moreira et al., 2009). Early stages of inflammation can occur through the activation of PRRs on many cells including lymphocytes, granulocytes, and endothelial cells (Faure et al., 2001; Muzio et al., 2000). Tissue necrosis induced by adjuvants as in ‘danger signal’ hypothesis advanced by (Matzinger, 1994), who proposed that apart from self/non-self discrimination against infection, danger signals from damaged cells (now referred to as “Damage signals” can trigger activation of the immune system through activation of inflammasome leading to release of proinflammatory cytokine such as IL-1 β . Molecules associated with tissue damage such as uric acid, nucleotides, ATP, reactive oxygen intermediates, and cytokines are released at the injection site due to tissue damage (Shi et al., 2003).

Recent evidence suggests that induction of inflammation and recruitment of immune cells to the site of injection are important mechanisms of action for PCEP as an adjuvant. Adjuvants can be expensive components in vaccines. If the dose of an adjuvant can be reduced without impacting vaccine efficacy, the results could be improved vaccine safety (less signs of inflammation at the site of injection) and reduced vaccine cost. We injected varying doses of PCEP [(high (500 µg) and medium (100 µg), and low (20 µg) into piglets and evaluated the inflammatory responses (granuloma formation, lymphocytes infiltration, epithelial necrosis, and suppurative inflammation) at the injection site and the draining lymph nodes and the antigen – specific immune responses when administered alone or as part of an swine influenza virus H1N1 vaccine. When PCEP was injected alone, inflammatory cells appeared at the injection site between 4 and 14 days after injection, dominated initially by neutrophils, followed by lymphocytes and later macrophages in all the PCEP doses. PCEP induced localized and controlled cell death in the skin, suggesting that the danger signals released from dead cells contributed to the enhanced immunogenicity. We suggest that PCEP appears to be responsible for the inflammatory responses at the site of injection that are critical in initiation of the immune responses even with reduced doses. Indeed, our recent studies have indicated that PCEP plays a role in the recruitment of neutrophils, natural killer cells, macrophages, eosinophils and immature dendritic cells to the site of injection and draining lymph nodes (Awate et al., 2012; Awate et al., 2014; Magiri et al., 2016) which in turn promotes the adaptive immune response to a vaccine (Magiri et al., 2018a).

In conclusion, we suggest that PCEP directly activates the innate immune responses at the site of injection. PCEP induced activation of innate immune responses at the injection site involves activation of adjuvant core response genes, production of cytokines and chemokines,

recruitment of various immune cells. All these events promote a strong immunocompetent environment at the injection site that may significantly contribute to the adjuvant activity of PCEP. In general, activation of innate immunity appears to be an important mechanism through which PCEP mediates its adjuvant activity. We propose a MOA model whereby PCEP induces tissue damage at the site of injection and the subsequent release of damage associated molecular patterns (DAMPs) which may activate the inflammasome and contribute to increased immunogenicity of the co-administered antigens.

Future directions.

The trend in vaccine development is moving away from the use of whole-cell or virus vaccines to subunit vaccines because of the long track record of safety of such vaccines. However, purified antigens often lack sufficient immunogenicity and therefore require addition of potent adjuvants to induce protective immune responses. The growing number of immune potentiators under investigation targets diverse innate immune mechanisms. Thus, the long-term goal should focus on selection of the optimal platforms and identification of key innate immune targets for induction of potent, but safe, immune responses.

We have demonstrated that PCEP induces expression of cytokine, chemokines and immune receptors genes, which then lead to secretion of pro-inflammatory cytokines at the site of injection thus causing inflammatory responses and immune cells recruitment. Further investigations are necessary to comprehend the diverse contribution of this immunostimulatory environment at the site of injection to the development of adaptive immune responses and identify the immune cells involved by flow cytometry

The contribution of the antigen to the inflammatory response still remains unclear. We have shown that PCEP induced acute but not chronic inflammatory responses at the injection site

and the draining lymph nodes when injected i.d. indicating that this adjuvant may be regarded as safe for use in this species. Further, When the PCEP dose was reduced, we generally observed significantly less histopathological changes without impacting the immune response to an influenza vaccine. There are many questions that need to be addressed. For example, does adjuvant activity of PCEP depend on the nature of antigen? Some antigens may have microbial components that have adjuvant activity, which may results in a synergistic effect hence increasing PCEP activity. Does the tissue damage caused by PCEP at the site of injection activate the inflammasome through DAMPs? And if the inflammasome is activated does it contribute to the adjuvant activity of PCEP? Are there other possible mechanisms involved in the adjuvant activity of PCEP? The mechanistic understanding of the innate immune system in regard to adjuvants and the tools to manipulate it are growing, and together these will make a significant impact on vaccine development in future.

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